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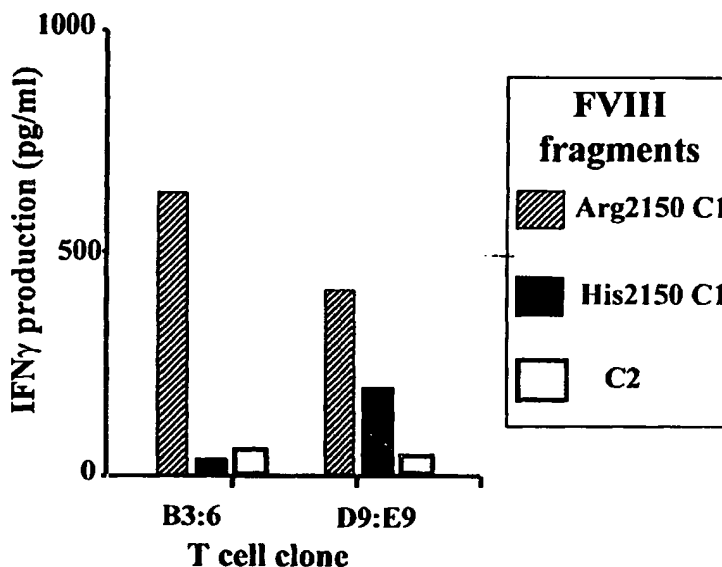
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(54) Title: **RECOMBINANT MOLECULES WITH REDUCED IMMUNOGENICITY, METHODS AND INTERMEDIATES FOR OBTAINING THEM AND THEIR USE IN PHARMACEUTICAL COMPOSITIONS AND DIAGNOSTIC TOOLS**



(57) Abstract: The invention provides a recombinant mammalian protein modified to eliminate or reduce by at least about 80 %, with respect to activation by the wild-type protein, the activation of at least one T-cell clone derived from a mammal with antibody against the wild-type protein, the said recombinant protein having, in its active form, a specific activity higher than 0.1, for use as a medicament. The recombinant protein is useful for making a pharmaceutical composition for the prevention or treatment of a disease induced by a lack or a dysfunction of a human protein, such as hemophilia.

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RECOMBINANT MOLECULES WITH REDUCED IMMUNOGENICITY, METHODS AND INTERMEDIATES FOR OBTAINING THEM AND THEIR USE IN PHARMACEUTICAL COMPOSITIONS AND DIAGNOSTIC TOOLS.

5 The present invention relates to recombinant molecules with reduced immunogenicity, methods and intermediates for producing them and therapeutic and diagnostic applications resulting therefrom. More particularly, this invention relates to recombinant mammalian proteins, e.g. coagulation factors, with reduced immunogenicity as active ingredients for the treatment of patients with diseases
10 induced by a dysfunction of a protein, in particular various types of hemophilia.

BACKGROUND OF THE INVENTION

Factor VIII (hereinafter referred as FVIII) is a protein providing important coagulant cofactor activity and is one of human clotting factors with a rather high molecular weight (about 265,000) and a very low normal plasma concentration
15 (0.0007 μ mole/litre). With its 2,332 amino-acid residues, FVIII is one of the longest known polypeptide chains and is synthesized in the liver, the spleen and the placenta. Its gene has been shown to include 186,000 nucleotides. FVIII circulates as inactive plasma protein. Factors V and VIII are homologous proteins sharing a common structural configuration of triplicated A domains and duplicated C domains
20 with structurally divergent B domains connecting the A2 and A3 domains.

The human FVIII gene was isolated and expressed in mammalian cells, as reported by various authors, including Wood et al. in *Nature* (1984) 312:330-337 and the amino-acid sequence was deduced from cDNA. U.S. Patent No. 4,965,199 discloses a recombinant DNA method for producing FVIII in mammalian host cells
25 and purification of human FVIII. The human FVIII detailed structure has been extensively investigated. The cDNA nucleotide sequence encoding human FVIII and predicted amino-acid sequence have been disclosed for instance in U.S. Patent No. 5,663,060. In a FVIII molecule, a domain may be defined as a continuous sequence of amino-acids that are defined by internal amino-acid
30 sequence homology and sites of proteolytic cleavage by a suitable protease such as thrombin. Unless otherwise specified, FVIII domains include the following

amino-acid residues, when the sequences are aligned with the human amino-acid sequence: A1, residues 1-372; A2, residues 373-740; B, residues 741-1648; A3, residues 1690-2019; C1, residues 2020-2172; C2, residues 2173-2332. The remaining sequence, residues 1649-1689, is usually referred to as the FVIII light chain activation peptide. FVIII is produced as a single polypeptide chain which, upon processing within the cell, is rapidly cleaved after secretion to form a heterodimer made of a heavy chain containing the A1, A2 and B domains and a light chain made of the A3-C1-C2 domains, according to Kaufman et al., *J. Biol. Chem.* (1988) 263:6352-6362. The two chains are non-covalently bound by divalent cations. Both the single-chain polypeptide and the heterodimer circulate in plasma as inactive precursors, as taught by Ganz et al., *Eur. J. Biochem.* (1988) 170:521-528. Activation of factor VIII in plasma initiates by thrombin cleavage between the A2 and B domains, which releases the B domain and results in a heavy chain consisting of the A1 and A2 domains, according to Eaton et al., *Biochemistry* (1986) 25:505-512. Human recombinant FVIII may be produced by genetic recombination in mammalian cells such as CHO (Chinese Hamster Ovary) cells, BHK (Baby Hamster Kidney) cells or other equivalent cells.

Pratt et al. in *Nature* (1999) 402:439-42 disclose the detailed structure of the carboxy-terminal C2 domain of human FVIII, which contains sites that are essential for its binding to von Willebrand factor and to negatively charged phospholipid surfaces. This structure, which reveals a beta-sandwich core from which two beta-turns and a loop display a group of solvent-exposed hydrophobic residues, partly explains mutations in the C2 region that lead to bleeding disorders in hemophilia A. According to Gale et al. in *Thromb. Haemost.* (2000) 83:78-85, of the at least 250 missense mutations that cause FVIII deficiency and hemophilia A, 34 are in the C domains.

Haemophilia A is a disease characterized by insufficient quantity of functionally active FVIII molecules. The disease, which affects 1/10,000 males, is classified according to the level of functional FVIII in severe (< than 1% FVIII), mild (1-5%) and moderate forms (>5%).

FVIII is a cofactor of the intrinsic pathway of the coagulation cascade, which acts by increasing the proteolytic activity of activated factor IX over factor X, in the so-called tenase complex formation. Patients suffering from hemophilia A present with bleedings which are either spontaneous in the severe form of the disease, or occur after trauma in the mild/moderate forms.

Hemophilia A patients are usually treated by replacement therapy, which consists in infusing human FVIII either purified from pools of donor plasma, or obtained by cDNA recombination technology. For instance, U.S. Patents No. 5,618,788 and No. 5,633,150 disclose producing functional species of human FVIII via recombinant DNA technology. In particular, they disclose (i) isolated DNA comprising a sequence encoding functional human FVIII, and (ii) a replicable expression vector capable, in a transfectant culture of cells, of expressing the said DNA sequence. They also express the idea that various human FVIII derivatives may potentially exist and be prepared by single or multiple amino acid deletions, substitutions, insertions or inversions, e.g. by means of site directed mutagenesis of the underlying DNA. These documents however do not disclose what these deletions, substitutions, insertions or inversions should be in order to have specific usefulness in diagnostic or therapeutic applications.

U.S. Patents No. 5,364,771 and No. 5,663,060 disclose purified hybrid factor VIII molecules comprising non-human mammalian and human amino acid sequences and having procoagulant activity *in vitro*. They also disclose compositions comprising such molecules combined with a pharmaceutically acceptable carrier, which are useful in treating human patients having antibodies to FVIII that inhibit coagulation activity. However these hybrid FVIII molecules suffer from the well known disadvantages of heterologous systems, i.e. although for instance porcine FVIII can be administered to humans with inhibitor antibodies, because there is only limited cross-reactivity between human and porcine FVIII and porcine FVIII is not inactivated by patient inhibitor antibodies, however tolerance to porcine FVIII is only transient and high titer antibodies towards both human and porcine FVIII can be detected in plasma of such patients after only a few days of

administration. This indicates that the repertoire of B lymphocytes contains cells able to produce antibodies recognizing porcine FVIII.

One of the major complications of the replacement therapy is the elicitation of an immune response towards the infused compound. Thus, antibodies to FVIII preclude further infusion, as exogenous FVIII is immediately neutralized by circulating anti-FVIII antibodies.

Different factors are known to be associated with the emergence of anti-FVIII antibodies, also called inhibitors. Thus, patients with the severe form of the disease are more prone to develop inhibitors than patients with the mild/moderate form. Some FVIII products have also been associated with a higher incidence of inhibitors. Altogether, about 25% of haemophilia A patients produce inhibitory anti-FVIII antibodies.

Factor IX (hereinafter referred as FIX) is a globular protein which has a molecular weight of about 70,000 daltons and which, in a normal individual, is constantly produced in the liver and circulates at a normal blood plasma concentration of about 5 µg/ml. FIX is a vitamin K-dependent protein which also participates in blood coagulation. It is synthesized in the form of a zymogen and undergoes three types of post-translational modifications before being secreted into the blood: (i) vitamin-K-dependent conversion of glutamic acid to carboxyglutamic acid, (ii) addition of hydrocarbon chains and (iii) beta-hydroxylation of an aspartic acid. It participates in the blood coagulation cascade and is used for the treatment of hemophilia B patients

Although FIX inhibitors are far less common and occur in about 2 to 3% of boys with hemophilia B (compared to an occurrence of about 30 to 50% of inhibitors in boys with hemophilia A), approximately half of such cases where inhibitors against FIX occur are accompanied by the occurrence of anaphylaxis or severe hypersensitivity reactions to any FIX-containing product, according to Lusher in *Best Pract. Res. Clin. Haematol.* (2000) 13:457-468.

Anti-FVIII antibodies are mostly immunoglobulin G (IgG) antibodies. The production of such antibodies is believed to be "T-cell dependent", which means

that help provided by specific T lymphocytes is required for efficient activation of B lymphocytes.

An immune response towards soluble antigens, such as FVIII, requires that the antigen is first processed by specialized cells, called antigen-presenting cells.

5 The function of such cells is precisely to present the antigen after processing to specific T cells. The latter can in turn help B lymphocytes to mature and secrete antibodies, i.e. specific T cell activation is a necessary step occurring before B cell activation. Therefore, preventing the activation of FVIII-specific T cells would also prevent B cell activation and thereby antibody production.

10 T cells recognize small stretches of amino acids presented in the context of class II major histocompatibility complex molecules (hereinafter referred as MHC-class II). In addition, T cell epitopes are organized according to a hierarchy consisting of immuno-dominant or major epitopes, minor epitopes and cryptic epitopes. Major epitopes are recognized by a majority of individuals and represent
15 the epitopes first recognized in the elicitation of an immune response. Eliminating such major T cell epitopes could therefore be sufficient to prevent T cell activation towards FVIII. Altogether, it is likely that only a limited number of T cells epitopes would need to be removed from the FVIII molecule in order to prevent the formation of inhibitory antibodies.

20 There are two methods by which a T cell epitope can be eliminated. This can first be achieved by the substitution of some amino-acids involved in the anchoring of the peptide containing the T cell epitope in major histocompatibility complexes at the surface of antigen-presenting cells. This first method results in a lack of peptide presentation, which is independent of the specificity of the T cell
25 receptor. The second method consists in altering the amino-acid residues that are directly involved in recognition by the T cell receptor.

Therefore there is a general need in the art for a method suitable to reduce the immune response of a protein, and more specifically to identify the T cell epitopes of proteins, in particular the T cell epitopes of blood coagulation factors
30 such as the FVIII molecule and factor IX, which are associated with the activation of T cells participating in the production of inhibitory antibodies. There is also a

need in the art for modified proteins that are able to substantially reduce the activation of T cells while keeping a high specific activity in replacement therapy. Apart from blood coagulation factors, a similar concern has been expressed for the enzyme replacement therapy developed for mucopolysaccharidose patients, since
5 immune responses have been reported in animal models and in human Gaucher patients. There is also a broad concern in the art that the development of antibodies to replaced proteins may limit the success of many human gene therapy approaches. A purpose of the present invention is to address these various needs.

SUMMARY OF THE INVENTION

10 A first aim of the present invention is a method to identify the T cell epitopes of proteins, in particular blood coagulation factors such as the FVIII molecule, which are associated with activation of T cells participating in the production of protein-specific antibodies, e.g. inhibitory antibodies. After effective performance of this identification method, amino-acids of such epitopes may then be substituted or
15 deleted in order to prevent T cell recognition. Amino-acid residues that are directly involved in the anchoring into MHC-class II determinants are preferably first identified and then substituted or deleted in order to prevent anchoring and thereby prevent T cell activation. Alternative possibilities will also be considered, which target amino-acid residues recognized by the T cell antigen receptor or flanking
20 residues involved in the affinity of T cell recognition. Residue substitutions and/or deletions which are able to preserve the major part of the protein biological activity, e.g. essentially all of the FVIII pro-coagulant function, are then selected. It is preferred if the said modification leaves the three-dimensional structure essentially unmodified, e.g. so as not to generate a new antibody binding site.

25 The invention thus provides a recombinant modified functional polypeptide which exerts at least one function of a mammalian protein and which eliminates or reduces by at least about 80%, with respect to activation by the unmodified polypeptide, the activation of at least one T-cell clone derived from a mammal with antibody against the said unmodified polypeptide, the said recombinant modified
30 functional polypeptide having *in vitro* a specific activity higher than 0.1, for use as a medicament.

Preferably, the T cell clone is derived from a human. More preferably, the said protein is a coagulation factor such as FVIII or FIX (or polymorphisms or variants thereof which have at least 10% of the catalytic activity of FVIII or FIX, respectively, in the coagulation cascade). More preferably, the recombinant polypeptide is in a purified form.

The invention also provides a method to generate a mammalian protein-specific T-cell clonal cell line by using a cell line expressing protein-specific antibodies, or fragments thereof, on its surface. Preferably the said cell line is a lymphoblastoid cell line. For instance, the invention provides T-cell clonal cell lines obtained by using as antigen-presenting cell the cell line KRIX 1. The cell line KRIX 1 was deposited by Dr. Marc Jacquemin (Center for Molecular and Vascular Biology, Herestraat 49, 3000 Leuven, Belgium) on July 1, 1999 at the Belgian Coordinated Collections of Microorganisms (BCCM), LMBP (plasmid collection, Laboratorium voor Moleculaire Biologie, Universiteit, K.L. Ledeganckstraat 35, 9000 Gent, Belgium) with Accession Number LMBP 5089CB. The cell line was assayed as being viable on July 9, 1999. Preferably, the protein involved in this method is a coagulation factor such as FVIII or FIX (or polymorphisms or variants thereof which have at least 10% of the catalytic activity of FVIII or FIX, respectively, in the coagulation cascade). More preferably, cell lines expressing the protein-specific antibodies are obtained by transfection or transduction with an expression vector for the protein-specific antibody or fragment thereof. More preferably the method comprises, in the following order, at least one of the following steps :

- identifying a peptide which has an epitope recognized by the clonal T-cell line by using a synthetic peptide library corresponding to the said protein,
- using the T cell clonal cell line to identify modifications of the T-cell epitope eliminating or reducing by at least about 80% its ability to activate the T-cell clonal cell line,
- producing a recombinant protein carrying a modification identified in the previous identification step,
- using the T-cell clone to verify that the modified protein does not provoke more than 20% T-cell activation by comparison to the wild-type protein, and

- controlling the activity of the modified protein by means of a suitable protein functional assay.

The present invention further encompasses a recombinant protein obtainable by this method, for instance a recombinant protein carrying, in respect of the wild-type protein, a substitution of a single residue and/or one amino-acid deletion. As a typical example thereof, this invention considers a recombinant human FVIII protein (or polymorphisms or variants thereof which have at least 10% of the catalytic activity of FVIII or FIX, respectively, in the coagulation cascade) carrying a substitution by another residue in the region between residues 2144 and 2161, for instance a substitution at residue 2153. This invention also relates to a recombinant protein carrying in respect of the wild-type protein several modifications located in one domain or combination of domains of the protein.

In another embodiment, this invention provides a peptide identified during the first step of the method as previously disclosed. Such a peptide preferably encompasses residues which can be mutated or deleted to eliminate or reduce by at least about 80% the activation of at least one T-cell clone activated by a wild-type protein. For instance, when the protein is a coagulation factor such as the human FVIII (or polymorphisms or variants thereof which have at least 10% of the catalytic activity of FVIII or FIX, respectively, in the coagulation cascade), the said peptide includes at least residues 2144 to 2161 thereof.

This invention also provides *in vitro* use of such a peptide for evaluating T-cell reactivity, and *in vivo* use of such a peptide for evaluating and/or modulating T-cell reactivity.

In still another embodiment, this invention provides a DNA sequence coding for a recombinant polypeptide such as previously defined, in particular the DNA sequence of human FVIII or of human FIX (or polymorphisms or variants thereof which have at least 10% of the catalytic activity of FVIII or FIX in the coagulation cascade). For example, one embodiment includes FVIII (or polymorphisms or variants thereof which have at least 10% of the catalytic activity of FVIII, in the coagulation cascade) carrying one single mutation at residue 2153. The invention

further relates to an expression vector including such a DNA sequence and a suitable promoter.

This invention also relates to a pharmaceutical composition comprising an effective amount of a recombinant protein, a peptide or an expression vector such as previously disclosed, and a pharmaceutically acceptable carrier. Finally, the invention provides a method for the prevention or treatment of a disease induced by a lack or a dysfunction of a human protein, comprising administering to a patient in need thereof an effective amount of a recombinant protein, a peptide or an expression vector such as previously disclosed. The said protein may be a coagulation factor such as FVIII or (the disease to be treated being hemophilia A) or FIX (the disease to be treated being hemophilia B).

Importantly, the present invention is widely applicable to any mutant or variant of the relevant protein, in particular to any mutant or variant of the FVIII or FIX molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows IFN- γ production following activation of T-cell clonal cell lines with human factor VIII.

Figure 2 shows stimulation of T-cell clonal cell lines with recombinant human factor VIII fragments.

Figure 3 shows the results of T-cell clone epitope mapping with synthetic peptides of the C1 domain of human factor VIII.

Figure 4 shows that non-specific B cell lines present human FVIII peptides but not intact FVIII.

Figure 5 shows that point mutations alter the T-cell epitope recognized by clonal cell lines.

DEFINITIONS

As used herein, "modified protein (or polypeptide)" denotes any recombinant protein (or polypeptide) molecules in which a single or a small number of amino-acids have been either substituted by any other amino-acid residue or deleted. Such amino-acid substitution or deletion can be located anywhere in the protein

molecule. It also denotes protein molecules in which amino-acid residues have been substituted and/or deleted at more than a single location. In the latter case, any combination of substitution and deletion can be considered.

The term "unmodified protein or polypeptide" relates generally to wild-type proteins or polypeptides having a normal function and includes polymorphisms which do not affect the catalytic activity sufficiently to generate a pathological state. Hence, wild-type, hybrid, truncations of a non functional domain (such as the B domain of factor VIII) or (single or multiple) point mutations (mutation being defined as substitution, deletion, insertion or inversion) are included within the scope of this term.

As used herein, "polymorphism" refers to the regular and simultaneous occurrence in a single interbreeding population of two or more alleles of a gene, where the frequency of the rarer alleles is greater, typically greater than 1%) than can be explained by recurrent mutation alone.

With respect to factor VIII, for example, a rest catalytic activity of at least 10% is considered not to generate a serious pathological state in humans.

"Non-autologous" with respect to humans means not from the same individual.

As used herein, the terms "epitope", "antigenic site" and "antigenic determinant" are used synonymously and are defined as a portion of the protein that is specifically recognized by an antibody or by the antigen receptor of a T cell. It can consist of any number of amino-acid residues and can be dependent upon the primary, secondary or tertiary structure of the protein. Thus, a protein that includes at least one epitope may be used as a reagent in the diagnostic assays.

As used herein, a "T cell epitope" is a stretch of amino-acids from the sequence of a protein and which varies in length between about 7 and 30 amino-acids. This epitope contains (i) residues enabling it to anchor into MHC class II molecules and (ii) distinct residues that are recognized by the antigen receptor of the corresponding T cell.

As used herein, a "B cell epitope" is a set of amino-acids that are present either as a sequence of amino-acids from a protein molecule, or as discontinuous

residues located at a distance from each other but brought together in the 3-D structure of the molecule. The B cell epitope is recognized by antibodies at the surface of specific B cells and by soluble antibodies produced by such B cells.

As used herein, "specific activity" makes reference to the ratio of the biological activity of a protein in a certain diagnostic assay to the biological activity of the corresponding wild-type protein in the same assay. For instance, as applied to FVIII, it refers to the activity that will correct the coagulation defect of human FVIII deficient plasma and is measured in units of clotting activity per milligram total FVIII protein in an assay in which the clotting time of human FVIII deficient plasma is compared to that of normal human plasma. In this assay, the shorter the time for clot formation, the greater the activity of the FVIII being assayed.

As used herein, "expression vector" means a vector which is capable of expressing a DNA sequence contained therein, where such sequence is operably linked to another sequence, such as a promoter, capable of effecting their expression. The expression vector replicates in the host cell, either by means of an intact operable origin of replication or by functional integration into the cell chromosome. In general, an expression vector useful in recombinant DNA technology is in the form of a "plasmid" which refers to circular double stranded DNA loops. However the invention includes all such other forms of expression vectors which serve equivalent functions.

DETAILED DESCRIPTION OF THE INVENTION

Although the present invention will be explained in details with respect to human FVIII, it is based on a concept widely applicable to mammalian proteins, in particular to blood coagulation factors, and therefore should not be interpreted solely in connection with human FVIII but should be extended to any protein of mammalian origin, which is known to be specifically involved in a specific disease. In addition the invention will mainly be described with reference to wild-type FVIII (or FIX) as an unmodified protein but the present invention also includes point mutations being introduced into polymorphisms of FVIII (or FIX). The fact that a polymorphism or a modified protein results in a mild disease state does not prevent

its use with the present invention (by further point modifications, for example) where the aim is to treat patients with a more serious disease state, that is the present invention aims at alleviate life-threatening states (while reducing the risk of a T-cell response to any such modified protein) but does not necessarily require
5 reinstatement of full functionality.

As briefly stated previously, the invention involves a method to produce a recombinant mammalian protein with reduced immunogenicity, comprising the steps of :

- 10 (a) isolating from a mammal antibody-producing T-cells against a wild-type mammalian protein,
- (b) identifying the major T-cell epitopes of the said wild-type mammalian protein which are associated with activation of T cells obtained in step (a), and
- (c) substituting or deleting amino-acids of the major T-cell epitopes identified in
15 step (b) in such way as to eliminate or reduce by at least about 80% T cell activation.

This method may further comprise the step of selecting the amino-acid substitutions or deletions of step (c) which are able to preserve a specific activity of the recombinant mammalian protein higher than about 0.1. Furthermore, the identifying step (b) may be performed by means of a peptide library from the wild-
20 type mammalian protein.

More preferably, this invention is applicable to proteins which (i) are in current use or have been suggested for use in a therapeutic treatment and (ii) give rise to the occurrence of inhibitory antibodies in the patient. A non limiting list of such proteins includes:

- 25 - proteins involved in diseases of the immune system such as the gene product of the FMF gene responsible for familial mediterranean fever, the interleukin-2 receptor gamma (IL2RG) gene or adenosine deaminase (ADA) gene in severe combined immunodeficiency;
- proteins involved in metabolic disorders such as phenylalanine hydroxylase
30 (PAH) in phenylketonuria, apolipoprotein E in Gauchers disease, ornithine ketoacid aminotransferase (OAT) in gyrate atrophy of the choroid coating

retina, ABC1 (ATP-binding cassette) in Tangier disease, beta-hexosaminidase A (HEXA) in Tay-Sachs disease;

- proteins involved in diseases of the musculature such as the cytoskeletal protein dystrophin in Duchenne muscular dystrophy, the survival motor neuron gene (SMN1) in spinal muscular atrophy (SMA);
- proteins involved in diseases of the nervous system such as the superoxide dismutase (SOD1) in amyotrophic lateral sclerosis (ALS), the gene product of the FMR1 gene in fragile X syndrome;
- proteins involved in diseases caused by a disfunction of cell signaling such as the gene product of the ATM gene in ataxia telangiectasia, the gene product GLC1A gene in glaucoma, the gene product PAX3 in Waardenburg syndrome (WS), WRN helicase in Werner's disorder;
- proteins involved in diseases caused by a disfunction of transporter proteins such as the gene product of the CFTR gene in cystic fibrosis, the copper transporter protein encoded by the ATP7B gene in Wilson's disease.

The skilled person will readily be able to take benefit from the invention by first determining the probability to be successful in performing the invention for a given protein with minimal investigation, i.e. without undue burden and time efforts, according to the following instructions. First, the skilled person will generate at least a T-cell clonal cell line reacting against the protein of interest, using methods standard in the art. In this respect, a representative collection of T cell clones must be established in order to identify as many T cell epitopes as possible. Then, a peptide library of the protein of interest will be prepared, using peptide synthesis procedures common in the art. Then, the first step of the method of the invention, i.e. identifying by means of the peptide library corresponding to the protein of interest at least one peptide which has an epitope recognized by the clonal T-cell line, is performed. After this step, it becomes possible to identify the peptide that generates T cell clone activation. From this information, the skilled person is able to (i) derive the number of epitopes involved and (ii) evaluate where such epitopes are located on the protein of interest. If the number of epitopes involved is higher than about 20, it is likely that the invention will require extensive investigations

which will make it less attractive. On the contrary, If the number of epitopes involved is not above about 20, then the invention will likely be helpful without an undue burden of effort and, therefore, the second step of the method of the invention, i.e. using the T cell clonal cell line to identify modifications of the T-cell epitope eliminating or reducing by at least about 80% its ability to activate the T-cell clonal cell line, will be performed. The skilled person readily understands that the amount of effort will depend not only upon the number of epitopes to be modified but also upon whether these are located in a region of the protein which is critical for its functional activity and/or its structural integrity.

10 The method disclosed in the present invention has advantages with respect to previous methods of generating recombinant molecules with reduced immunogenecity and where peptides are presented to T cells (WO 01/40281 or WO 99/53038). This method can not be followed to identify relevant T cell epitopes in proteins such as Factor VIII. Reding et al. in (Reding et al (2000) 15 Thromb. Haemost. 84, 643-652) studied the response to FVIII peptides of polyclonal CD4+ T cells purified either from the blood of normal individuals, or from haemophilia A patients with or without anti FVIII antibodies. These authors demonstrated that healthy individuals and haemophilia A patients without inhibitor have polyclonal T cells recognising several of the peptides derived from the 20 sequence of the FVIII molecule, although these subjects are tolerant to FVIII. Moreover, when polyclonal CD4+ T cells derived from haemophilia A patients with inhibitor were assayed with the same FVIII peptides, FVIII-specific T cells were identified, which recognised identical peptides as those recognised by healthy individuals or haemophilia A patients without inhibitor. Therefor all healthy subjects 25 have FVIII specific T cells identifiable by a method where peptides are presented to T cells, although these individuals remain completely tolerant towards their own FVIII and can be transfused with blood or plasma without developing an immune response towards FVIII. The reason for the detection of T cells recognising FVIII peptides in healthy individuals or haemophilia A patients without inhibitor is still 30 unknown. It is possible that the T cell proliferation assay detects T cells recognising peptides which are normally not presented to T cells by antigen

presenting cells loaded with the native protein. The processing of a protein within the late endosome occurs through a number sequential events involving proteolytic cleavages, reduction of disulphide bridges and selection of the amino acid sequences that fir best for binding to major histocompatibility complex (MHC) molecules. The result is that only a small number of T cell epitopes are selected for presentation, even with proteins of relatively large size. By contrast, a short peptide, whenever processed by an antigen-presenting cell, finds no competition with other potential epitopes present in the corresponding native protein. In the absence of such competition, the peptide is presented even when its affinity for MHC-class II binding is not high. Besides, short peptides of the size used in publications such as Reding et al. are able to bind directly to MHC class II molecules at the surface of the antigen presenting cells and can be recognised by T cells without need for processing. Moreover, the size of the natural T cell repertoire in man is such that is able to recognise virtually any possible epitope. Consequently, all epitopes presented by MHC class II molecules loaded with synthetic peptides will be matched by a corresponding T cell.

Mild/moderate haemophilia A patients rarely produce inhibitory antibodies towards infused FVIII. This is due to the lower frequency of FVIII administration in such patients, who suffer from bleedings only after trauma or during surgery. However, this low frequency of inhibitors is also due to the fact that such patients having significant levels of circulating FVIII had the opportunity to become immunologically unresponsive against FVIII. The mechanisms by which such unresponsiveness develops are well known to those skilled in the art and can be found in recently published reviews such as *Immunological Tolerance* (1998) Novartis Foundation Symposium 215, Wiley, Chichester. Briefly, during the ontogeny of the immune system, lymphocytes capable to recognize and to be activated by self antigens are eliminated from the repertoire by deletion mechanisms occurring in the bone marrow (B cells) and in the thymus (T cells).

Patients with mild/moderate haemophilia A are therefore expected to have become tolerant towards all FVIII T cell epitopes, except those which are affected by the mutation/deletion responsible for the haemophilia phenotype. If an inhibitor

antibody is formed, then the T cells required for antibody production should be located close to or at the said mutated/deleted site. In support of this, epidemiological studies in mild/moderate haemophilia A patients have identified a preferential association of inhibitory antibodies with mutations located within discrete regions of the FVIII molecule, such as parts of the C1 and of the C2 domains, as reported by Hay et al., *Thromb.Haemost.* (1998) 79: 762.

From peripheral blood lymphocytes of patients with inhibitor and mild/moderate haemophilia A, it is possible to expand the population of FVIII-specific T cells which are potentially involved in the production of antibodies. Such T cells can be cloned by methods well known in the art. However, we discovered that efficient characterization of such T cell lines required the use of FVIII-specific B cell lines as antigen-presenting cells.

The following is an example of a method to generate and identify cell lines expressing coagulation factor antibodies at their surface. Antigen-presenting cells activate CD4+ T cells that recognize peptides bound to MHC class II molecules. For efficient activation, T cells require peptide presentation in the context of self MHC class II molecules. Therefore, cell lines expressing FVIII-specific surface immunoglobulins should express MHC class II molecules identical to those expressed by the individual from whom FVIII-specific T cell clones are to be derived. Such cell lines can be obtained by first immortalizing the patient's B lymphocytes with the Epstein-Barr virus, according to methods well known by those skilled in the art, for instance as disclosed by Jacquemin et al. in *Blood* (1998) 92:496. In a second step, FVIII-specific B cells are selected by screening cell culture supernatants for the presence of anti-FVIII antibodies. Antibodies towards FVIII are identified by incubating cell culture supernatants in microtitration polystyrene plates coated with FVIII. The binding of specific antibodies is detected by addition of an anti-human IgG reagent coupled to an enzyme. Addition of an enzyme substrate which is converted to a colored compound in the presence of the enzyme allows the detection of specific antibodies. Such method referred to as enzyme-linked immunoassay (ELISA) is well known to those skilled in the art and described in details e.g. in *Current Protocols in Immunology*, chapter 2, John Wiley

& Sons (1994). Microcultures producing anti-FVIII antibodies are then expanded and cloned. Clonal cell lines are then further selected for the production of anti-FVIII antibodies, e.g. according to Jacquemin et al. (cited *supra*).

Alternatively, cell lines which do not produce anti-FVIII antibodies can be transformed in order to let them express FVIII-specific antibodies, provided that the selected cell lines express MHC class II molecules identical to the patients from whom T lymphocytes are taken for cloning. Such cell lines can be obtained by first immortalizing patient's B lymphocytes with the Epstein-Barr virus. In a second step, the cell lines are transformed with an expression vector coding for a FVIII-specific immunoglobulin. For this purpose, the complete cDNA encoding the heavy and light chains of a FVIII specific antibody is cloned using the cDNA obtained from a FVIII-specific cell line. Human lymphoblastoid cell lines (Jacquemin et al. cited *supra*) or mouse hybridomas (according to Gilles et al. in *Blood* (1993) 82:2452) producing anti-FVIII antibodies can be used as a source of such cDNA. The introduction of the gene coding for the anti-FVIII antibody can then be carried out by transfection or transduction of the target cell. In order to prepare DNA for transfection, the complete cDNA encoding the heavy and light chains of the FVIII-specific antibody is cloned in a eukaryotic expression vector designed for the independent expression of two genes from a single plasmid, such as pBudCEA4.0 (available from Invitrogen, Groningen, The Netherlands). Importantly, the heavy chain must contain the transmembrane and intracytoplasmic portions of surface immunoglobulin. These portions can be cloned by polymerase chain reaction using cDNA of a lymphoblastoid cell line such as BO2C11 (Jacquemin et al. cited *supra*) by methods well known by those skilled in the art. CHO cells are then transfected with the expression vector. Microcultures containing cells producing anti-FVIII antibodies are then expanded and cloned. Clonal cell lines are then further selected for the production of anti-FVIII antibodies. Cell surface expression of the anti-FVIII antibody can be detected by labelling the cells with FVIII labelled with biotin or a fluorescent dye using techniques well known by those skilled in the art, as taught e.g. by Current Protocols in Immunology, Chapter 5, John Wiley & Sons, Inc. (1994).

Alternatively, the target cell can be transduced with a gene coding for a FVIII-specific antibody using a viral vector. For example, a full length heavy and light chain cDNA, including the cytoplasmic and transmembrane domains, is inserted into a linearized retrovirus vector. The plasmid encoding the retroviral
5 vector and heavy and light chains is then transfected by calcium-phosphate precipitation into a packaging cell line. Supernatant from virus-producing cells are then filtered and tested for the presence of replication competent virus. The target cell line (for example a patient's lymphoblastoid cell line immortalized with the Epstein-Barr virus) expressing the correct MHC class II molecule is then
10 transduced either by co-cultivation with the retrovirus packaging cell line or by retroviral supernatant infection, as is well known by those skilled in the art, e.g. by Moreau-Gaudry et al. in *Blood* (1995) 85:1449.

After generating suitable T cell clones, these are then used to map precisely the corresponding epitope. The corresponding amino-acid residues are then
15 modified (i.e. mutated and/or deleted) in the full-length FVIII molecule by using methods well known in the art to identify the amino-acid residues which are critical for interaction with T cells. As a quality control measure, the resulting FVIII mutant molecules are then assessed for reactivity with a panel of T cell clones directed towards the region where the mutation/deletion has been introduced.

20 The production of antibodies towards soluble antigens requires that the antigen is first presented to the immune system in a manner suitable for recognition. Specialized cells, called antigen-presenting cells (hereinafter referred as APC) have the function to absorb the antigen, digest it by a combination of hydrolytic enzymes before associating the digested antigen fragments with
25 molecules of the MHC class II complexes. The resulting complexes are then migrating to the surface of the cells for presentation to T cells. MHC class II complex molecules are constituted of a cleft that is open on both sides, allowing peptides of various lengths to bind. Such binding involves usually four or five major amino-acid residues that interact directly with corresponding residues in the MHC
30 molecule via hydrophobic and electrostatic attractions. The anchoring residues are not contiguous but rather located two or three amino-acids apart. Examples of such

anchoring residues can be found e.g. in Hammer et al., *J. Exp. Med.* (1994) 180:2353-8. Such anchoring residues can to some extent be predicted using available algorithms such as Tepitope (Raddrizzani et al., *Briefings in Informatics* (2000) 1:179-189), but can also be determined by *in vitro* experiments, using for
5 instance competitive binding assays on soluble MHC molecules as disclosed for instance by Wall et al, *J. Immunol.* (1994) 152:4526.

The function of the anchoring residues is to maintain the T cell epitope in a fixed conformation, which is then recognizable by the antigen receptor of a T cell. The latter recognizes mostly a conformation made by both residues of the T cell
10 epitope, but also by side residues of the MHC molecule. Some degree of flexibility exists in the actual amino-acid residues recognized by T cells: limited amino-acid substitution can be carried out with no loss of T cell receptor binding, provided that the overall three-dimensional conformation is maintained.

The avidity of T cells for such peptide-MHC class II molecules complex is
15 relatively low. This is compensated by the recognition of multiple peptides at the surface of the APC by T cell receptors. The interaction between APC and T cells is further reinforced by mutual recognition of a number of complementary surface molecules, such as CD40-CD40L, CD80/CD86 and CD28, the CD4 molecule, etc. Detailed description of these events can be found for instance in Lenschow et al.,
20 *Annu. Rev. Immunol.* (1996) 14:233-258 and in Oxenius et al., *Adv. Immunol.* (1998) 70:313-367. Following recognition of a peptide, the T cell undergoes a number of activation events starting by the phosphorylation of cytoplasmic proteins coupled to the T cell receptor. One of the consequences of such activation is the production of soluble mediators called interleukins, which participate in the tuning
25 of B cell functions.

B cells, by contrast with T cells, recognize the antigen directly through their specific surface immunoglobulins. B cells are the main APC for secondary and subsequent immune responses, while professional APC (macrophages or dendritic cells) are the main APC for primary responses, when no specific B cells of
30 sufficient avidity for the antigen are present. Upon re-exposure to the antigen, the latter is internalized by B cells via surface immunoglobulins, which deliver the

antigen into the late endosomal compartment for processing and binding to MHC class II molecules, much alike professional APC. The cognate interactions between specific T cells and the B cell presenting the antigen is followed by activation of the T cell, with the aim of providing B cells with the signals necessary for full activation and maturation into antibody-producing cells. In the absence of such interaction
5 between B and T cells, and therefore in the absence of specific T cells, no antibody production is elicited.

Hemophilia A is a hereditary disease characterized by the lack or insufficient function of FVIII. Patients suffering from such disease are usually treated by
10 infusion of purified FVIII obtained by plasma purification or by genetically-engineered mammalian cells transfected to produce FVIII. One major complication of FVIII infusion is the elicitation of a specific immune response towards infused FVIII. This immune response includes the production of high-affinity IgG antibodies, of which some inhibit the function of FVIII. The production of such
15 inhibitor antibodies requires the presence of specific T cells, which provide B cells with the necessary signals to mature into antibody-secreting plasmocytes. The involvement of T lymphocytes in the development of antibodies against FVIII was shown by Singer et al. in *Thromb. Haemost.* (1996) 76:17-22 and by Reding et al. in *Thromb. Haemost.* (2000) 84: 643-52.

20 A more complete answer to the question of the presence of FVIII-specific T cells in haemophilia A patients is provided by the present invention. Thus, T cell clones have been derived from peripheral lymphocytes of a patient suffering from mild haemophilia and with an inhibitor antibody to wild-type FVIII, as described in more details in Example 1 below.

25 Antibodies to FVIII belong to the immunoglobulin-G (IgG) class, with only rare exceptions. Such IgG antibodies are of high affinity, suggesting that their production occurs only in a context of help provided by specific T cells. It is indeed well known by those skilled in the art that the production of antibodies with high affinity to soluble antigens is the result of a T cell driven maturation of the immune
30 response in the presence of the antigen. A general overview of the mechanisms sustaining the production of antibodies can be found in Fanning et al., *Clin.*

Immunol. Immunopathol. (1996) 79:1-14. Further evidence for T cell dependency is suggested by the observation that a high proportion of anti-FVIII antibodies belong to the IgG4 sub-type, the selection of which is known to be strictly T cell dependent.

5 Taken together, these data provide evidence that the production of anti-FVIII antibodies is dependent on the presence of FVIII-specific T cells and therefore that preventing or switching off specific T cell activation will with high probability result in prevention or suppression of the production of anti-FVIII antibodies.

 Since FVIII is a molecule of high molecular weight, the number of possible T
10 cell epitopes is large. This renders the identification of pathogenic epitopes, namely the epitopes that activate T cells participating with B cells in the production of inhibitory antibodies, rather elusive. This is well illustrated by the findings of Reding et al. (cited *supra*), who identified a large number of peptides scattered over the entire length of FVIII and able to activate T cells. The present inventors have now
15 discovered a method suitable to identify relevant, pathogenic T cell epitopes, comprising deriving corresponding T cells from the blood of patients with haemophilia A developing an immune response towards exogenous FVIII. In the following examples, the effectiveness of the method of the invention is demonstrated by using cells from patients with mild/moderate forms of haemophilia
20 A recognizing only exogenous FVIII, however the method can be applied to any patient developing anti-FVIII antibodies.

 FVIII is a normal constituent of the coagulation pathway and as such belongs to self proteins. In order to prevent the emergence of an immune response against self-constituents and auto-immunity, nature has developed highly efficient
25 means by which the immune system is depleted of cells (B and T lymphocytes) with reactivity towards self components. Thus, one of the functions of the thymus is to select and sort out the T cell repertoire so as to eliminate T cells reacting against self proteins. Detailed description of the different mechanisms at play in the thymus can be read e.g. in Plum et al., *Ann. NY Acad. Sci.* (2000) 917: 724-731.

30 T cell epitopes are organized according to a hierarchy including major, minor and cryptic epitopes. Cryptic epitopes are not processed and presented to T

cells when the full-length protein is used. Such cryptic epitopes can however be detected when peptides from the protein are used instead. Under such conditions, peptides can be efficiently processed and therefore presented by APC to T cells. Because of lack of presentation in the thymus during the selection of the T cell repertoire, T cells recognizing cryptic epitopes are not eliminated and can be found in the periphery. Hence, peripheral blood T cells can be found which proliferate when presented with peptides of the corresponding proteins (see Reding et al., cited *supra*). Major and minor T cell epitopes are presented during thymus selection, which results in the elimination of corresponding T cells.

In mild/moderate hemophilia A, patients have significant levels of circulating FVIII. This FVIII is almost identical to wild-type FVIII, except for the region where the mutation/deletion is located. Thymus selection in such patients is therefore operating normally for all T cells reacting towards major and minor determinants, except for those determinants that are altered by the mutation/deletion itself. Such patients therefore risk developing an immune response to FVIII when exposed to normal wild-type FVIII used for replacement therapy. The immune response is then directed only to wild-type FVIII and not towards the patient's self-FVIII.

The mutation/deletion occurring in the FVIII gene in mild/moderate hemophilia A patients can be easily identified by those skilled in the art. Methods to carry out such identification can be found in Higuchi, *Proc. Nat. Acad. Sci.* (1991) 88:8307-8311. When an immune response develops towards wild-type FVIII in mild/moderate hemophilia A patients, it should be primarily directed towards the region of FVIII containing the mutation/deletion. If the plasma contains anti-FVIII antibodies, the peripheral blood of such patients should contain FVIII-specific T cells which activate FVIII-specific B cells to produce anti-FVIII antibodies.

FVIII-specific T cells from mild/moderate hemophilia A patients having inhibitory antibodies towards wild-type FVIII therefore represent a unique source of material to select pathogenic T cells. Such T cells can be expanded and cloned by techniques well-known in the art, and which are described in more details in Example 1 below. T cell clones can be used to determine the precise epitope recognized and identify amino-acid residues involved in either MHC class II

anchoring or T cell receptor recognition. This can be carried out using methods such as T cell activation with series of peptides with sequence varying by only one amino-acid at a time from the wild-type sequence. The basic principles of these methods are known in the art and are illustrated in Example 2 below. In addition,
5 residues binding to MHC class II molecules can be identified using purified MHC molecules of different haplotypes and inhibition of binding assays, as reported by Wall et al. (cited *supra*).

Amino-acid residues involved in the binding of the T cell epitope in MHC class II molecules are then mutated/deleted in the cDNA coding for FVIII.
10 Mammalian cells such as Chinese Hamster Ovary (CHO) cells or Baby Hamster Kidney (BHK) cells are then transfected with mutated/deleted cDNA for production of a new mutated FVIII molecule. The latter is then tested for lack of activation of T cell clones and for the presence of co-factor activity in the coagulation cascade. Methods to introduce a mutation or delete DNA base pairs are well known in the
15 art, as well as methods used to transfect mammalian cells and to produce recombinant FVIII molecules. Such methods can be found in general methodology reviews such as Current Protocols in Molecular Biology and Current Protocols in Protein Science (Wiley & Sons, Inc.). Example 2 below provides further details on such methods. The functional activity of the resulting modified FVIII molecule is
20 then tested in either coagulation assays or chromogenic assays as described by Jacquemin et al. in *Blood* (2000) 96:958. Particularly preferred are mutations which do not disturb three-dimensional structure of the protein sufficiently to create a new binding site for antibodies. This may be described as a "conservative substitution".

Reduced immunogenicity of the modified FVIII molecules of the present
25 invention can be assessed in different systems. For example, an immunodeficient mouse strain can be used, the immune system of which is reconstituted with immunocompetent cells of human origin. Thus, severe combined immunodeficiency mice (hereinafter referred as SCID) can be reconstituted by intraperitoneal injection of peripheral blood lymphocytes of a haemophilia A patient
30 presenting with an inhibitor. Such lymphocytes include immunocompetent B cells for antibody production and T cells to provide the necessary help to B cells in the

production of anti-FVIII inhibitory antibodies. Series of mice can be reconstituted with cells of a single donor. Some of such mice are then immunized with wild-type FVIII, against which the patient is producing inhibitor antibodies, which results in the production of inhibitor antibodies. These can be detected by standard inhibition of coagulation assays well known in the art. Another group of mice is immunized with the newly produced mutated FVIII molecule. In such case, it can be demonstrated whether a reduced response or no antibody response is elicited. Methods for the use of SCID mice are readily available, for instance from Vanzielegem et al., *Thromb. Haemost.* (2000) 83:833-9.

The invention also provides a method by which T cell epitopes can be identified in other proteins of the coagulation cascade which are known to elicit immune responses altering the normal physiological activity of such protein. One particular example of such protein is FIX. Antibodies to FIX have been described in patients suffering from hemophilia B, which is due to a lack or insufficient function of FIX, as disclosed by Lusher in *Semin. Thromb. Haemost.* (2000) 26:179. The latter is an enzyme which activates factor X in the coagulation cascade. The invention therefore further provides a method, along the lines disclosed hereinabove, to produce new recombinant FIX molecules for replacement therapy in patients suffering from hemophilia B.

The invention further provides a method to identify pathogenic T cell epitopes in other blood coagulation proteins such as factor X, factor V, factor VII, protein S and protein C. Antibodies inhibiting the function of such proteins have been described in Kunkel, *Hematol. Oncol. Clin. North Am.* (1992) 6:1341-1357.

As previously indicated, the recombinant human proteins and expression vectors of the invention are useful as therapeutically active ingredients for the manufacture of pharmaceutical compositions comprising the same and further comprising at least one pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" as used herein means any material or substance with which the active ingredient is formulated in order to facilitate its application or dissemination to the locus to be treated, for instance by dissolving, dispersing or diffusing the said composition, and/or to facilitate its storage,

transport or handling without impairing its effectiveness. The pharmaceutically acceptable carrier may be a solid or a liquid or a gas which has been compressed to form a liquid, i.e. the compositions of this invention can suitably be used as concentrates, emulsions, solutions, granulates, dusts, sprays, aerosols, suspensions, ointments, creams, tablets, pellets or powders.

Suitable pharmaceutical carriers for use in the said pharmaceutical compositions and their formulation are well known to those skilled in the art, and there is no particular restriction to their selection within the present invention. In particular, they include other human proteins, e.g. human serum albumin, as described for example in Remington's Pharmaceutical Sciences by E.W. Martin.

The pharmaceutical compositions of the invention may also include other additives such as wetting agents, dispersing agents, stickers, adhesives, emulsifying agents, solvents, coatings, antibacterial and antifungal agents (for example phenol, sorbic acid, chlorobutanol), isotonic agents (such as sugars or sodium chloride) and the like, provided the same are consistent with pharmaceutical practice, i.e. carriers and additives which do not create permanent damage to mammals, in particular to humans. The pharmaceutical compositions of the present invention may be prepared in any known manner, for instance by homogeneously mixing, coating and/or grinding the active ingredient, in a one-step or multi-steps procedure, with the selected carrier material and, where appropriate, the other additives such as surface-active agents. They may also be prepared by micronization, for instance in view to obtain them in the form of microspheres usually having a diameter of about 1 to 10 μm , namely for the manufacture of microcapsules for controlled or sustained release of the active ingredients.

Suitable surface-active agents to be used in the pharmaceutical compositions of the present invention are non-ionic, cationic and/or anionic materials having good emulsifying, dispersing and/or wetting properties. Suitable anionic surfactants include both water-soluble soaps and water-soluble synthetic surface-active agents. Suitable soaps are alkaline or alkaline-earth metal salts, unsubstituted or substituted ammonium salts of higher fatty acids ($\text{C}_{10}\text{-C}_{22}$), e.g. the sodium or potassium salts of oleic or stearic acid, or of natural fatty acid

mixtures obtainable from coconut oil or tallow oil. Synthetic surfactants include sodium or calcium salts of polyacrylic acids; fatty sulphonates and sulphates; sulphonated benzimidazole derivatives and alkylarylsulphonates. Fatty sulphonates or sulphates are usually in the form of alkaline or alkaline-earth metal salts, unsubstituted ammonium salts or ammonium salts substituted with an alkyl or acyl radical having from 8 to 22 carbon atoms, e.g. the sodium or calcium salt of lignosulphonic acid or dodecylsulphonic acid or a mixture of fatty alcohol sulphates obtained from natural fatty acids, alkaline or alkaline-earth metal salts of sulphuric or sulphonic acid esters (such as sodium lauryl sulphate) and sulphonic acids of fatty alcohol/ethylene oxide adducts. Suitable sulphonated benzimidazole derivatives preferably contain 8 to 22 carbon atoms. Examples of alkylarylsulphonates are the sodium, calcium or alkanolamine salts of dodecylbenzene sulphonic acid or dibutyl-naphthalenesulphonic acid or a naphthalene-sulphonic acid/formaldehyde condensation product. Also suitable are the corresponding phosphates, e.g. salts of phosphoric acid ester and an adduct of p-nonylphenol with ethylene and/or propylene oxide, or phospholipids. Suitable phospholipids for this purpose are the natural (originating from animal or plant cells) or synthetic phospholipids of the cephalin or lecithin type such as e.g. phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerine, lysolecithin, cardiolipin, dioctanoylphosphatidyl-choline, dipalmitoylphosphatidyl -choline and their mixtures.

Suitable non-ionic surfactants include polyethoxylated and polypropoxylated derivatives of alkylphenols, fatty alcohols, fatty acids, aliphatic amines or amides containing at least 12 carbon atoms in the molecule, alkylarenesulphonates and dialkylsulphosuccinates, such as polyglycol ether derivatives of aliphatic and cycloaliphatic alcohols, saturated and unsaturated fatty acids and alkylphenols, said derivatives preferably containing 3 to 10 glycol ether groups and 8 to 20 carbon atoms in the (aliphatic) hydrocarbon moiety and 6 to 18 carbon atoms in the alkyl moiety of the alkylphenol. Further suitable non-ionic surfactants are water-soluble adducts of polyethylene oxide with polypropylene glycol, ethylenediaminopolypropylene glycol containing 1 to 10 carbon atoms in the alkyl

chain, which adducts contain 20 to 250 ethyleneglycol ether groups and/or 10 to 100 propyleneglycol ether groups. Such compounds usually contain from 1 to 5 ethyleneglycol units per propyleneglycol unit. Representative examples of non-ionic surfactants are nonylphenol -polyethoxyethanol, castor oil polyglycolic ethers, polypropylene/polyethylene oxide adducts, tributylphenoxypolyethoxyethanol, polyethyleneglycol and octylphenoxypolyethoxyethanol. Fatty acid esters of polyethylene sorbitan (such as polyoxyethylene sorbitan trioleate), glycerol, sorbitan, sucrose and pentaerythritol are also suitable non-ionic surfactants.

Suitable cationic surfactants include quaternary ammonium salts, preferably halides, having 4 hydrocarbon radicals optionally substituted with halo, phenyl, substituted phenyl or hydroxy; for instance quaternary ammonium salts containing as N-substituent at least one C₈-C₂₂ alkyl radical (e.g. cetyl, lauryl, palmityl, myristyl, oleyl and the like) and, as further substituents, unsubstituted or halogenated lower alkyl, benzyl and/or hydroxy-lower alkyl radicals.

A more detailed description of surface-active agents suitable for this purpose may be found for instance in "McCutcheon's Detergents and Emulsifiers Annual" (MC Publishing Corp., Ridgewood, New Jersey, 1981), "Tensid-Taschenbuch", 2nd ed. (Hanser Verlag, Vienna, 1981) and "Encyclopaedia of Surfactants (Chemical Publishing Co., New York, 1981).

Pharmaceutical forms suitable for injectionable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation thereof. Typical carriers for this purpose therefore include biocompatible aqueous buffers, ethanol, glycerol, propylene glycol, polyethylene glycol and the like, and mixtures thereof.

The present invention also provides the use of a human recombinant protein as a medicament. When the said protein is a blood coagulation factor, it is preferably used for preventing and/or treating disorders of hemostasis, in particular, coagulation disorders and other thrombotic pathologic conditions in mammals, preferably in humans. When the said protein is not a blood coagulation factor, but a protein involved in another biological process such as disclosed herein-above, it may be used for the treatment or prevention of diseases, a non-exhaustive list of

which was given herein-before in connection with examples of relevant proteins. The said recombinant protein may be provided to a patient by any means well known in the art, i.e. orally, intranasally, subcutaneously, intramuscularly, intradermally, intravenously, intraarterially, parenterally or by catheterization. The administration can be for prolonged periods, such as chronic administration and/or repetitive administration. The said modified protein can be used for treatment of patients with a deficiency in the wild type protein and wherein administration of the wild type protein causes side effects such as anaphylactic shock as is known for e.g. with Factor IX.

As an example, when human FVIII is the protein involved, the recombinant protein of the invention will usually be administered intravenously, in an amount of 40 units (1 unit FVIII being defined as the amount of FVIII present in 1 ml of a pool of plasma of normal individuals) per kg body weight of the patient for pre-operative indications, 15 to 20 units per kg body weight for minor bleeding episodes, and 20 to 40 units per kg body weight every day for maintenance dose or prophylaxis. For the treatment of patients who develop inhibitors to FVIII, doses up to 200 units per kg body weight may be administered twice a day.

The following examples are provided for the purpose of illustrating the FVIII embodiment of the present invention and should in no way be understood as limiting the scope of this invention which, as previously indicated, is widely applicable to a broad range of proteins, including blood coagulation factors.

EXAMPLE 1 - Determination of the T cell epitope recognised by FVIII-specific T cell clones.

A) expansion of FVIII-specific oligoclonal T cell lines

Peripheral blood mononuclear cells (PBMC) are purified by Lymphoprep density gradient centrifugation (available from Nycomed Pharma). In order to generate autologous dendritic cells, PBMC are depleted from T lymphocytes using CD4 and CD8 MicroBeads (available from Miltenyi Biotec), following the manufacturer's recommendations. The negatively selected cells were incubated for two hours at 37°C in culture flasks (Falcon) at a density of 2×10^6 cells/ml in Roswell Park

Memorial Institute (hereinafter referred as RPMI) 1640 medium supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine and 1% autologous plasma previously heated at 56°C for 30 minutes, according to methods well known by those skilled in the art (e.g. Chaux et al. in *J. Exp. Med.* (1999) 189:767). Non-adherent cells are frozen in liquid nitrogen and used as a source of B lymphocytes. Adherent cells are cultured in the presence of interleukin-4 (hereinafter IL-4) (100 U/ml) and granulocyte macrophage-colony stimulating factor (hereinafter referred as GM-CSF) (100 ng/ml) in RPMI-1% autologous serum. Half of the medium was replaced on day 2 and day 4 with fresh medium plus interleukins.

On day 5, autologous dendritic cells were incubated at 37°C, 5% CO₂ for 18 hours in RPMI-1% autologous serum supplemented with IL-4 (100 u/ml), GM-CSF (100 ng/ml), and tumor necrosis factor-alpha (hereinafter TNF-α) (1 ng/ml) in the presence of 20 µg/ml plasma-derived or recombinant FVIII. Cells were washed and added at 10⁴ per well to 10⁵ CD4⁺ lymphocytes (isolated using CD4 MicroBeads as above) in 200 µl Iscove's modified Dulbecco's medium (hereinafter IMDM) (available from Gibco BRL) supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine and 10% human serum in the presence of IL-6 (1.000 U/ml) and IL-12 (10 ng/ml). The CD4⁺ lymphocytes were re-stimulated on day 7, day 14 and day 21 with autologous dendritic cells freshly loaded with FVIII and were grown in IMDM-10% human serum supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). The cells containing proliferating CD4⁺ T cells were assessed on day 35 for the presence of FVIII-specific T cells.

B) detection of FVIII-specific oligoclonal T cells by IFN-γ production

Autologous Epstein-Barr virus immortalized B cells were incubated for 18 hours in the presence of 20 µg/ml of plasma-derived FVIII. FVIII-pulsed lymphoblastoid cells were washed and incubated at 5,000 per well in round bottomed microculture plates with 5,000 CD4⁺ T cells in IMDM-10% human serum supplemented with IL-2 (25 U/ml). After 20 hours, the supernatants were collected

and interferon-gamma (hereinafter IFN- γ) in the supernatant was measured by an ELISA assay using reagents from Medgenix Diagnostics Biosource.

C) isolation of CD4⁺ T cell clones

The cell lines that recognized cells loaded with FVIII were cloned by limiting
5 dilution, using as stimulating cells FVIII-specific autologous lymphoblastoid cells
(from the cell line KRIX 1 deposited with the Belgian Coordinated Collections of
Micro-organisms under accession number LMBP 5089CB) loaded with FVIII by
incubation for 18 hours in the presence of 20 μ g/ml FVIII. Allogeneic cells were
added as feeders cells. Established clonal CD4⁺ T cell lines were then grown in
10 complete IMDM-10% human serum supplemented with IL-2 (50 U/ml), IL-7 (5
ng/ml) and 0.5 μ g/ml phytohemagglutinin (HA 16, available from Murex
Diagnostics) according to well known methods (e.g. Chaux et al., cited *supra*).
Using these methods, two T cell clones, named hereinafter B3:6 and D9:E9, were
isolated.

15 The cell line D9:E9 was deposited by Dr. Marc Jacquemin (Center for Molecular
and Vascular Biology, Herestraat 49, 3000 Leuven, Belgium) on April 25, 2002 at
the Belgian Coordinated Collections of Microorganisms (BCCM), LMBP (plasmid
collection, Laboratorium voor Moleculaire Biologie, Universiteit, K.L.
Ledeganckstraat 35, 9000 Gent, Belgium) with Accession Number LMBP 5850CB.

20 The cells were assayed as being viable on May 17, 2002.

D) characterization of T cell clones

The procedure of paragraph 1-B above was repeated. As shown in Figure 1,
when three T cell clones are incubated with autologous lymphoblastoid cell lines
25 expressing FVIII-specific surface immunoglobulin (KRIX 1) and loaded with FVIII,
high amounts of IFN- γ (1,200-1,600 pg/ml) are detected in culture supernatant.
This response is specific for FVIII as indicated by the 10-times lower production of
IFN- γ in the absence of FVIII. Similar results were obtained with recombinant FVIII,
indicating that T cells were specific for FVIII and not for other contaminant proteins
30 present in FVIII concentrates purified from plasma.

In order to determine the location of the T cell epitopes in the FVIII molecule, T cells are incubated with recombinant fragments corresponding to different domains of the FVIII molecule and produced in *E.Coli* according to Jacquemin et al. in *Blood* (1998) 92:496-506. As shown in Figure 2, only the native C1 domain induces secretion of IFN- γ by the two T cell clones. No IFN- γ secretion is induced using the C2 domain (Figure 2) or other recombinant fragments corresponding to the A1, A2 or A3 domains (data not shown). The C1 domain carrying substitution Arg2150 \rightarrow His does not stimulate IFN- γ production by B3:6. However IFN- γ is produced following incubation with D9:E9, although IFN- γ concentration remains lower than with the native C1 domain. These data demonstrate that substitution of Arg2150 by His eliminates a T cell epitope recognized by B3:6 but only partially alters the epitope recognized by the T cell clone D9:E9. These data suggest that the T cell epitopes recognized by the two T cell clones are located in the C1 domain and that they include amino-acid residue 2150.

E) mapping of the T cell epitopes using T cell clones and synthetic peptides

The T cell epitope is further defined using synthetic peptides encompassing residue 2150. As shown in Figure 3, a peptide encompassing residues 2144-2161 stimulates the T cell clones B3:6 and D9:E9. The epitope specificity of the T cell clones is further defined using shorter synthetic peptides. A peptide encompassing residues 2148-2161 stimulates B3:6, but not D9:E9, indicating that the specificity of the latter is different. The synthetic peptide encompassing residues 2144-2158 also stimulates both T cell clones, as shown in Figure 3.

F) Comparison of FVIII-specific and non FVIII-specific lymphoblastoid cell lines as antigen-presenting cells

FVIII-specific B cell lines are used as antigen-presenting cells during cloning of FVIII-specific T cells. This significantly differs from previously published methods (e.g. Chaux et al. cited *supra*) known to generate specific T cell clones directed toward proteins other than FVIII, in which non-specific autologous antigen-presenting cells were used. Prior art indeed indicates that an antibody can alter antigen processing, particularly when the antibody is directed towards a region that

also contains a T cell epitope. Synthetic peptides were efficiently presented by FVIII-specific and non-specific lymphoblastoid cell lines, as shown by figure 4, which indicated that these cells expressed the correct MHC class II molecules. These unexpected results indicated that FVIII-specific cell lines should preferably
5 be used to generate and clone FVIII-specific T cells when the epitope of the T cell clone is unknown and complete FVIII molecule must be used as an antigen.

EXAMPLE 2 - identification of substitutions altering T cell epitopes

The activation of the T cell clones by the wild type FVIII peptide 2144-2161 was compared with activation in the presence of the synthetic peptide 2144-2161
10 carrying the substitution of arginine by histidine at residue 2150. No production of IFN- γ was detected when the T cell clone B3:6 was stimulated with the said synthetic peptide. The T cell clone D9:E9 produced a significant amount of IFN- γ following stimulation with the mutated peptide, although the response was reduced by comparison to that observed with the wild type peptide, as shown in figure 5.
15 The fact that substitution Arg2150His completely prevents activation of T cell clone B3:6 but only partially that of clone D9:E9 is in agreement with the observation that these two T cell clones recognize different epitopes, as already shown in figure 3. It also indicates that a FVIII molecule carrying this substitution will have a significant, but only partial, antigenicity for haemophilia A patients T cells. This
20 observation is also in agreement with the observation that several patients carrying the substitution Arg2150His who developed an immune response towards exogenous FVIII also develop an immune response towards their own FVIII according to Santagostino et al., *Thromb. Haemost.* (1995) 74:619.

Similarly, the analysis of other synthetic peptides carrying point mutations
25 indicates that substitution Thr2154Ile prevents recognition by B3:6 but not by D9:E9 (as shown in figure 5), also in agreement with the observation that these two T cell clones recognize different epitopes. Unexpectedly, the substitution Pro2153Gln completely prevents recognition by both T cell clones (as shown in figure 5). Similar results are obtained using a recombinant FVIII molecule carrying
30 the substitution Pro2153Gln.

Interestingly, analysis of the activity of a FVIII molecule carrying the substitution Pro2153Gln indicates that its pro-coagulant activity is close to normal, as shown below.

These observations indicate that FVIII molecules carrying the substitution Pro2153Gln have a completely abrogated antigenicity with regards to the T cell clones B3:6 and D9:E9.

Given the T-cell dependency of the development of humoral response towards FVIII, the administration of such a mutated FVIII molecule to haemophilia A patients will result in a reduced propensity to develop such a humoral response.

10 EXAMPLE 3 - production and characterization of recombinant mutated FVIII.

A) plasmid mutagenesis

Mutagenesis is performed within a mammalian expression vector coding for the FVIII cDNA, according to Vehar et al. in *Nature* (1984) 312:337. Also suitable for this purpose is the vector encoding the B domain-less FVIII cDNA according to Lind et al., *Eur. J. Biochem.* (1995) 232:19-27. Mutant plasmids are generated through oligonucleotide site-directed mutagenesis utilizing the polymerase chain reaction in accordance with Jacquemin et al. in *Blood* (2000) 92:496. Codon No. 2150 was mutated from CGT to CAT, predicting an arginine to histidine amino-acid change. Codon No. 2153 was mutated from CCA to CAA, predicting an amino acid change from Pro to Gln. Codon No. 2201 was deleted. All mutated cDNAs are controlled by sequencing in both directions using a Genetic Analyzer 3.10 from Perkin Elmer.

B) Chinese hamster ovary (CHO) cell transfection

In order to establish cell lines expressing wild-type or mutant FVIII, CHO cells are transfected with plasmid encoding wild-type or mutated FVIII using FUGENE 6 (Boehringer Mannheim, Brussels, Belgium) according to the manufacturer's instructions.

C) FVIII production by transfected CHO cells

Briefly, CHO cells (8×10^4 cells/well) were seeded in 6-well plates (Life Technologies) using minimal essential medium alpha (hereinafter MEM- α ,

available from Life Technologies Ltd., Paisley, United Kingdom) supplemented with 10% fetal calf serum (hereinafter FCS). After 24 hours incubation, a transfection mixture of 0.5 µg DNA in 10 µl of tris-ethylenediamine tetraacetic acid (EDTA), 100 µl OPTIMEM and 2 µl FUGENE® 6 (available from Roche) was applied to the cells. After 48 hours, the cells were washed twice with MEM-α and the culture medium was replaced by MEM-α supplemented with Nutridoma-CS® (Boehringer Mannheim, Germany) and 3 mM sodium butyrate. After 16 hours, the conditioned medium was harvested, centrifuged to remove cell debris and assayed for FVIII activity. The cell lines producing the highest FVIII amounts are expanded and sub-cloned twice.

D) evaluation of the specific activity of the FVIII molecule

The cofactor activity of recombinant FVIII molecules is evaluated using the FVIII chromogenic assay available from Dade AG (Switzerland) according to the manufacturer's recommendations. In this assay, thrombin-activated FVIII accelerates the conversion of factor X into factor Xa in the presence of factor IXa, phospholipids (hereinafter PL) and calcium ions; factor Xa activity is then assessed by hydrolysis of a p-nitroanilide substrate. Reagents, which were reconstituted according to the manufacturer's instruction, comprised bovine factor X (1 mM), factor IXa (0.3 mM) and thrombin (0.3 mM); CaCl₂ (30 mM), PL (60 mM), a chromogenic factor Xa substrate (CH₃OCO-D-CHG-gly-Arg-pNA:AcOH; 3.4 mM) and a thrombin inhibitor (L-amidinophenylalanine piperidine) according to Jacquemin et al. in *Blood* (1998) 92:496.

Plasma FVIII antigen levels were measured in ELISA using the Immunozyg FVIII:Ag® test (available from Immuno AG, Vienna, Austria) following the manufacturer's recommendations. Recombinant FVIII antigen levels were also measured in ELISA according to published methods, using monoclonal antibodies F4H12 or F15B12 recognizing the A1 or A2 domain of FVIII respectively, as capture antibodies. Bound FVIII was detected by the addition of a mixture of monoclonal antibodies 13, F8D6, F29A1 and F14A12, according to Jacquemin et al., *Blood* (2000) 96:962.

The FVIII specific activity is calculated as indicated before under the heading "Definitions". Dysfunctional FVIII molecules with specific activity as low as 0.01 were reported for FVIII variants, also called cross-reactive material positive FVIII in order to emphasize the disparity between the amount of FVIII protein and the level of FVIII functional activity, by Pemberton et al. in *Blood* (1997) 89:2413. Interestingly, the substitution Pro2153Gln results in a FVIII molecule with a specific activity close to normal. Similarly, we demonstrated that deletion of a single amino-acid residue, such as amino-acid 2201 in the FVIII C2 domain resulted in a FVIII molecule with a specific activity close to 1. This result is quite unexpected because this residue is located close to residues 2199 and 2200 which are known to mediate FVIII binding to phospholipids, an essential feature of FVIII, according to Pratt et al. in *Nature* (2000) 402:439-42.

Deletion of a single amino-acid residue, even in a region important for FVIII functional activity such as the C2 domain where this residue is located, can therefore be considered as a suitable alternative to the substitution of a defined residue. The surprising observations made with the Pro2153Gln substituted FVIII molecule indicate that it is possible to produce recombinant FVIII molecules which are not recognized by all T cell clones available so far while preserving a FVIII activity sufficient to guarantee haemostasis. Accordingly, these FVIII molecules are less immunogenic because T cell clones recognizing this epitope will not be activated following administration.

CLAIMS

1. A recombinant modified functional polypeptide which exerts at least one function of a mammalian protein and which eliminates or reduces by at least
5 about 80%, with respect to activation by the unmodified polypeptide, the activation of at least one T-cell clone derived from a mammal with antibody against the said unmodified polypeptide, the said recombinant modified functional polypeptide having *in vitro* a specific activity higher than 0.1, for use as a medicament, said functional polypeptide being selected from polypeptides
10 involved in blood coagulation, polypeptides involved in diseases of the immune system, polypeptides involved in metabolic disorders, polypeptides involved in diseases of the musculature, polypeptides involved in diseases of the nervous system and polypeptides involved in diseases caused by a dysfunction of cell signalling or transporter proteins.
15
2. A recombinant multiple point-mutated functional polypeptide which exerts at least one function of a mammalian protein and which eliminates or reduces by at least about 80%, with respect to activation by the unmodified polypeptide, the activation of at least one T-cell clone derived from a mammal with antibody
20 against the said unmodified polypeptide, the said recombinant modified functional polypeptide having *in vitro* a specific activity higher than 0.1, said functional polypeptide being selected from polypeptides involved in blood coagulation, polypeptides involved in diseases of the immune system, polypeptides involved in metabolic disorders, polypeptides involved in diseases
25 of the musculature, polypeptides involved in diseases of the nervous system and polypeptides involved in diseases caused by a dysfunction of cell signalling or transporter proteins.
3. A recombinant multiple point-mutated functional polypeptide according to claim
30 2, wherein the multiple point-mutations include (i) at least one point-mutation responsible for T-cell activation reduction or elimination and (ii) at least one

substitution of at least one other amino-acid residue resulting from an exonic polymorphism of the gene encoding the unmodified polypeptide.

4. A recombinant multiple point-mutated functional polypeptide according to claim 3, wherein the point-mutation (i) does not induce a conformational change of the unmodified polypeptide.
5. A recombinant multiple point-mutated functional polypeptide according to claim 3 or claim 4, wherein the amino-acid residue substitution (ii) does not influence the biological activity of the said recombinant polypeptide.
6. A recombinant multiple point-mutated functional polypeptide according to any of claims 3 to 5, wherein the amino-acid residue substitution (ii) does not induce the raising of antibodies against the said recombinant polypeptide.
7. A recombinant multiple point-mutated functional polypeptide according to any of claims 3 to 5, wherein the amino-acid residue substitution (ii) induces the raising of antibodies against the said recombinant polypeptide which do not affect its biological activity.
8. A recombinant multiple point-mutated functional polypeptide according to any of claims 2 to 7, for use as a medicament.
9. A recombinant functional polypeptide according to any of claims 1 to 8, for repetitive , i.e. chronic, administration to a human.
10. A recombinant functional polypeptide according to any of claims 1 to 9, for administration to a human without causing the side effects (e.g. anaphylactic shock) of the unmodified polypeptide.

- 11.A recombinant functional polypeptide according to any of claims 1 to 10, for administration to a human without eliciting antibodies affecting the biological activity of the said recombinant functional polypeptide.
- 5 12.A recombinant functional polypeptide according to any of claims 1 to 11, wherein the T cell clone is derived from a human.
- 13.A recombinant functional polypeptide according to claim 12 wherein the cell clone is the T cell clonal cell line D9:E9 deposited at BCCM with Accession
10 Number LMBP 5850CB.
- 14.A recombinant functional polypeptide according to any of claims 1 to 13, wherein the said unmodified polypeptide is a coagulation factor.
- 15 15.A recombinant functional polypeptide according to any of claims 1 to 14, for treating a thrombotic disorder.
- 16.A recombinant functional polypeptide according to any of claims 1 to 15, wherein the said unmodified polypeptide exerts the activity of a factor VIII
20 molecule.
- 17.A recombinant functional polypeptide according to claim 16, for treating haemophilia A.
- 25 18.A recombinant functional polypeptide according to claim 16 or claim 17, being a multiple point-mutated polypeptide derived from the unmodified factor VIII molecule by including (i) one point-mutation responsible for T-cell activation reduction or elimination and (ii) at least one substitution of at least one other amino-acid residue resulting from an exonic polymorphism of the gene
30 encoding the unmodified factor VIII molecule.

19. A recombinant functional polypeptide according to claim 18, wherein the point-mutation (i) does not induce a conformational change of the unmodified polypeptide.
- 5 20.A recombinant functional polypeptide according to claim 18 or claim 19, wherein the point mutation (i) lies in the light chain of the factor VIII molecule.
- 21.A recombinant functional polypeptide according to any of claims 18 to 20, wherein the point mutation (i) is in the C1 domain of the factor VIII molecule.
- 10 22.A recombinant functional polypeptide according to any of claims 18 to 21, wherein the point mutation (i) is in the region between residues 2144 and 2161 of the factor VIII molecule.
- 15 23.A recombinant functional polypeptide according to any of claims 18 to 22, wherein the point-mutation (i) is a Pro2153Gln substitution.
- 24.A recombinant functional polypeptide according to any of claims 18 to 23, wherein the amino-acid residue substitution (ii) is selected from the group
20 consisting of Asp56Val, Asp1241Glu, Leu1462Pro, Val2223Met and Met2238Val.
- 25 25.A recombinant functional polypeptide according to claim 16 or claim 17, being human Factor VIII with the substitution P2153Q as presented in SEQ ID NO:13.
26. A DNA nucleotide sequence presented in SEQ ID NO:18 encoding the polypeptide of claim 25.
- 30 27.A recombinant functional polypeptide according to claim 16 or claim 17, which eliminates or reduces by at least about 80%, with respect to activation by the

unmodified polypeptide, the activation of at least two T-cell clones selected from the repertoire of T-cell clones of patients with mild haemophilia, moderate haemophilia or severe haemophilia.

5 28.A recombinant functional polypeptide according to any of claims 1 to 15, wherein the said unmodified polypeptide exerts the function of a factor IX molecule.

29.A recombinant functional polypeptide according to claim 28, for treating
10 haemophilia B.

30.A recombinant functional polypeptide according to any of claims 1 to 13, wherein the said unmodified polypeptide is selected from proteins involved in blood coagulation, proteins involved in diseases of the immune system,
15 proteins involved in metabolic disorders, proteins involved in diseases of the musculature, proteins involved in diseases of the nervous system and proteins involved in diseases caused by a dysfunction of cell signalling or transporter proteins.

20 31.A method to generate a mammalian protein-specific T-cell clonal cell line by using a cell line expressing protein-specific antibodies, or fragments thereof, on its surface.

32.A method according to claim 31, wherein the cell line is a lymphoblastoid cell
25 line.

33.A method according to claim 32 wherein the cell line is the cell line KRIX 1 deposited with the Belgian Coordinated Collections of Micro-organisms with Accession Number LMBP 5089CB.

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34.A method according to claim 31 or claim 32, wherein the said protein is a coagulation factor.

5 35.A method according to claim 34, wherein the said coagulation factor is factor VIII.

36.A method according to claim 35, wherein the protein-specific T-cell clonal cell line is specific for the C1 domain of the factor VIII molecule.

10 37. A method according to claim 36 wherein the cell line is the T cell clonal cell line D9:E9 deposited at BCCM with Accession Number LMBP 5850CB.

38.A method according to claim 34, wherein the said coagulation factor is factor IX.

15 39.A method according to claim 31 or claim 32, wherein the said protein is selected from proteins involved in blood coagulation, proteins involved in diseases of the immune system, proteins involved in metabolic disorders, proteins involved in diseases of the musculature, proteins involved in diseases of the nervous system and proteins involved in diseases caused by a
20 dysfunction of cell signalling or transporter proteins.

40.A method according to any of claims 31 to 39, wherein cell lines expressing the protein-specific antibodies are obtained by transfection or transduction with an expression vector for the protein-specific antibody or fragment thereof.

25

41.A method according to any of claims 31 to 40, comprising the step of identifying a peptide which has an epitope recognized by the clonal T-cell line by using a synthetic peptide library corresponding to the said protein.

42. A method according to claim 41, further comprising the step of using the T cell clonal cell line to identify modifications of the T-cell epitope eliminating or reducing by at least about 80% its ability to activate the T-cell clonal cell line.

5 43. A method according to claim 42, further comprising the step of producing a recombinant protein carrying a modification identified in the previous identification step.

10 44. A method according to claim 43, further comprising the step of using the T-cell clone to verify that the modified protein does not provoke more than 20% T-cell activation by comparison to the wild-type protein.

15 45. A method according to claim 44, further comprising the step of controlling the activity of the modified protein by means of a suitable protein functional assay.

46. A recombinant functional polypeptide according to any of claims 1 to 30, being obtainable by a method according to any of claims 31 to 45.

20 47. A biologically active recombinant protein obtainable from a recombinant functional polypeptide according to any of claims 1 to 30.

48. A biologically active recombinant protein according to claim 47, being obtained by *in vitro* processing the recombinant functional polypeptide by means of a suitable protease.

25 49. A biologically active recombinant protein according to claim 47, being a factor VIII molecule obtained by *in vitro* processing the recombinant factor VIII by means of thrombin.

50. A biologically active recombinant protein according to claim 49, wherein the B domain of factor VIII has been deleted or replaced with a protease-cleavable amino-acid sequence.

5 51. A biologically active recombinant protein according to any of claims 47 to 50 for use as a medicament.

52. A peptide identified by using a T-cell clonal cell line generated by a method according to any of claims 31 to 41.

10

53. *In vitro* use of a peptide according to claim 52 for evaluating T-cell reactivity.

54. *In vivo* use of a peptide according to claim 52 for evaluating and/or modulating T-cell reactivity.

15

55. A DNA sequence coding for a recombinant multiple point-mutated functional polypeptide according to any of claims 2 to 7.

20

56. An expression vector including a DNA sequence according to claim 55 and a suitable promoter.

25

57. A pharmaceutical composition comprising an effective amount of a recombinant functional polypeptide according to any of claims 1 to 30 or an expression vector according to claim 56, and a pharmaceutically acceptable carrier.

30

58. A method of prevention or treatment of a disease induced by a lack or a dysfunction of a human protein, comprising administering to a human in need thereof an effective amount of a recombinant functional polypeptide according to any of claims 1 to 30 or an expression vector according to claim 52, said human protein being selected from proteins involved in blood coagulation, proteins involved in diseases of the immune system, proteins involved in

metabolic disorders, proteins involved in diseases of the musculature, proteins involved in diseases of the nervous system and proteins involved in diseases caused by a dysfunction of cell signalling or transporter proteins.

5 59.A method of prevention or treatment according to claim 58, wherein the said protein is a coagulation factor and the disease is a thrombotic disorder.

60.A method of prevention or treatment according to claim 58 or claim 59, wherein the said coagulation factor is factor VIII and the said disease is haemophilia A.

10

61.A method of prevention or treatment according to claim 58 or claim 59, wherein the said coagulation factor is factor IX and the said disease is haemophilia B.

15 62.A method of prevention or treatment according to claim 58, wherein the said protein is selected from proteins involved in blood coagulation, proteins involved in diseases of the immune system, proteins involved in metabolic disorders, proteins involved in diseases of the musculature, proteins involved in diseases of the nervous system and proteins involved in diseases caused by a dysfunction of cell signalling or transporter proteins.

20

63.A T-cell clonal cell line generated by a method according to any of claims 31 to 45.

25 64.A T-cell clonal cell line according to claim 63, being obtained by using as antigen presenting cell the cell line KRIX 1 deposited with the Belgian Coordinated Collections of Micro-organisms under accession number LMBP 5089CB.

30 65.A method to produce a recombinant therapeutically active modified polypeptide with reduced immunogenicity, the said polypeptide exerting at least one function of a mammalian protein, comprising the steps of :

45

- (a) generating from a mammal T-cell clones against a non-autologous protein which gives rise to the occurrence of inhibitory antibodies in a patient,
(b) identifying the major T-cell epitopes of the said wild-type therapeutically active mammalian protein which are associated with activation of T cell clones obtained in step (a), and
5 (c) mutating amino-acids of the major T-cell epitopes identified in step (b) in such a way as to eliminate or reduce by at least about 80% T cell activation.

66.A method according to claim 65, further comprising selecting the amino-acid mutations of step (c) which are able to preserve a specific activity of the recombinant therapeutically active polypeptide higher than about 0,1.

67.A method according to claim 65 or claim 66, wherein the identifying step (b) is performed by means of a peptide library from the unmodified polypeptide.

15 68.A method according to any of claims 65 to 67, wherein the mammalian protein is a human protein.

69.A method according to any of claims 65 to 68, wherein the mammalian protein is a coagulation factor.

70.A method according to any of claims 65 to 69, wherein the mammalian protein is factor VIII.

25 71.A method according to any of claims 65 to 69, wherein the mammalian protein is factor IX.

72.A method according to any of claims 65 to 68, wherein the mammalian protein is selected from proteins involved in diseases of the immune system, proteins involved in metabolic disorders, proteins involved in diseases of the musculature, proteins involved in diseases of the nervous system and proteins

30

involved in diseases caused by a dysfunction of cell signaling or transporter proteins.

73.A recombinant functional polypeptide according to any of claims 1 to 30,
5 wherein reduced T-cell activation results from immuno-dominant T-cell epitopes.

74.A recombinant functional polypeptide according to claim 73, wherein said
immuno-dominant T-cell epitopes are identifiable by means of step (b) of the
10 method of claim 65.

75.A recombinant multiple point-mutated factor VIII light chain molecule containing
the A3, C1 and C2 domains of factor VIII and being able to reconstitute a
biologically active recombinant factor VIII molecule when suitably processed
15 together with a mammalian or hybrid factor VIII heavy chain molecule
containing the A1 and A2 domains of factor VIII, wherein the immunogenicity of
the biologically active factor VIII molecule is reduced by providing, in respect of
the unmodified factor VIII light chain molecule, (i) at least one point-mutation in
a function-specific domain of the said light chain molecule, the said point-
20 mutation(s) being able to eliminate or reduce by at least about 80%, with
respect to activation by the unmodified factor VIII, the activation of at least one
T-cell clone derived from a mammal with antibody against the said unmodified
factor VIII, and the said point-mutation(s) further being such that the specific
activity of the biologically active recombinant factor VIII molecule is higher than
25 0.1, and (ii) at least one substitution of at least one other amino-acid residue
resulting from an exonic polymorphism of the gene encoding the unmodified
factor VIII light chain molecule.

76.A recombinant multiple point-mutated factor VIII light chain molecule according
30 to claim 75, wherein the substitution (ii) is selected from Val2223Met and
Met2238Val.

77.A recombinant multiple point-mutated factor VIII light chain molecule according to claim 75 or claim 76, wherein the point-mutation (i) is in the C1 domain of the light chain.

5

78.A recombinant multiple point-mutated factor VIII light chain molecule according to any of claims 75 to 76, wherein the point-mutation (i) is in the region between residues 2144 and 2161 of the unmodified factor VIII.

10 79.A recombinant multiple point-mutated factor VIII light chain molecule according to any of claims 75 to 78, being the amino-acid sequence as shown in SEQ ID NO: 15

15 80.A DNA sequence coding for a recombinant multiple point-mutated factor VIII light chain molecule according to any of claims 75 to 79.

81.A DNA sequence according to claim 80, being the nucleotide sequence as shown in SEQ. ID NO 14

20 82.An expression vector including a DNA sequence according to claim 80 or claim 81 and a suitable promoter.

83.An expression vector according to claim 82, belonging to the class of recombinant adeno-associated viral vectors.

25

84.An expression vector according to claim 82 or claim 83 which, upon co-infection of cells with a vector expressing the DNA sequence coding for the mammalian or hybrid factor VIII heavy chain molecule, is able to produce biologically active recombinant factor VIII in plasma.

30

85.A kit comprising :

48

(a) a first component being a recombinant multiple point-mutated factor VIII light chain molecule according to claim 75, and

(b) a second component being a mammalian or hybrid factor VIII heavy chain molecule which is able to reconstitute a biologically active recombinant factor VIII molecule when suitably processed in the presence of the first component.

86. A pharmaceutical composition comprising an effective amount of a recombinant multiple point-mutated factor VIII light chain molecule according to any of claims 75 to 79 or an expression vector according to any of claims 82 to 84, and a pharmaceutically acceptable carrier.

87. A method of prevention or treatment of a disease induced by a lack or a dysfunction of human factor VIII, comprising administering to a human in need thereof an effective amount of a recombinant multiple point-mutated factor VIII light chain molecule according to any of claims 75 to 79 or an expression vector according to any of claims 79 to 84 or a pharmaceutical composition according to claim 86.

88. A method according to claim 87, wherein the said disease is haemophilia A.

89. A recombinant point-mutated factor VIII light chain molecule containing the A3, C1 and C2 domains of factor VIII and being able to reconstitute a biologically active recombinant factor VIII molecule when suitably processed together with a mammalian or hybrid factor VIII heavy chain molecule containing the A1 and A2 domains of factor VIII, wherein the factor VIII clearance in plasma is reduced by providing, in respect of the unmodified factor VIII light chain molecule, at least one amino-acid deletion in the region between residues 2180 to 2220 of the C2 domain of the unmodified factor VIII.

90. A recombinant point-mutated factor VIII light chain molecule according to claim 89, being the amino-acid sequence as shown in SEQ ID NO:17
91. A DNA sequence coding for a recombinant point-mutated factor VIII light chain molecule according to claim 89 or claim 90.
92. A DNA sequence according to claim 91, being the nucleotide sequence as shown in SEQ ID NO:16
93. A biologically active recombinant factor VIII molecule comprising a recombinant point-mutated factor VIII light chain molecule according to claim 89 or claim 90 together with a mammalian or hybrid factor VIII heavy chain molecule containing the A1 and A2 domains of factor VIII.
94. A biologically active recombinant factor VIII molecule according to claim 93, further comprising the B domain factor VIII.
95. A biologically active recombinant factor VIII molecule according to claim 93 or claim 94, for use as a medicament.
96. A biologically active recombinant factor VIII molecule according to any of claims 93 to 95, for use in the treatment of haemophilia A.
97. A biologically active recombinant factor VIII molecule according to any of claims 93 to 96, for administration to a human.
98. A biologically active recombinant factor VIII molecule according to any of claims 93 to 97, for administration at a frequency less than one-third of the frequency of administration of the unmodified factor VIII molecule.

99. An expression vector including a DNA sequence according to claim 91 or claim 92 and a suitable promoter.
100. An expression vector according to claim 99, belonging to the class of recombinant adeno-associated viral vectors.
101. An expression vector according to claim 99 or claim 100 which, upon co-infection of cells with a vector expressing the DNA sequence coding for the mammalian or hybrid factor VIII heavy chain molecule, is able to produce biologically active recombinant factor VIII in plasma.
102. A kit comprising :
- (a) a first component being a recombinant point-mutated factor VIII light chain molecule according to claim 89 or claim 90, and
 - (b) a second component being a mammalian or hybrid factor VIII heavy chain molecule which is able to reconstitute a biologically active recombinant factor VIII molecule when suitably processed in the presence of the first component.
103. A pharmaceutical composition comprising an effective amount of a recombinant point-mutated factor VIII light chain molecule according to claim 89 or claim 90 or an expression vector according to any of claims 99 to 101, and a pharmaceutically acceptable carrier.
104. A method of prevention or treatment of a disease induced by a lack or a dysfunction of human factor VIII, comprising administering to a human in need thereof an effective amount of a recombinant point-mutated factor VIII light chain molecule according to claim 89 or claim 90, or an expression vector according to any of claims 99 to 101 or a pharmaceutical composition according to claim 103.

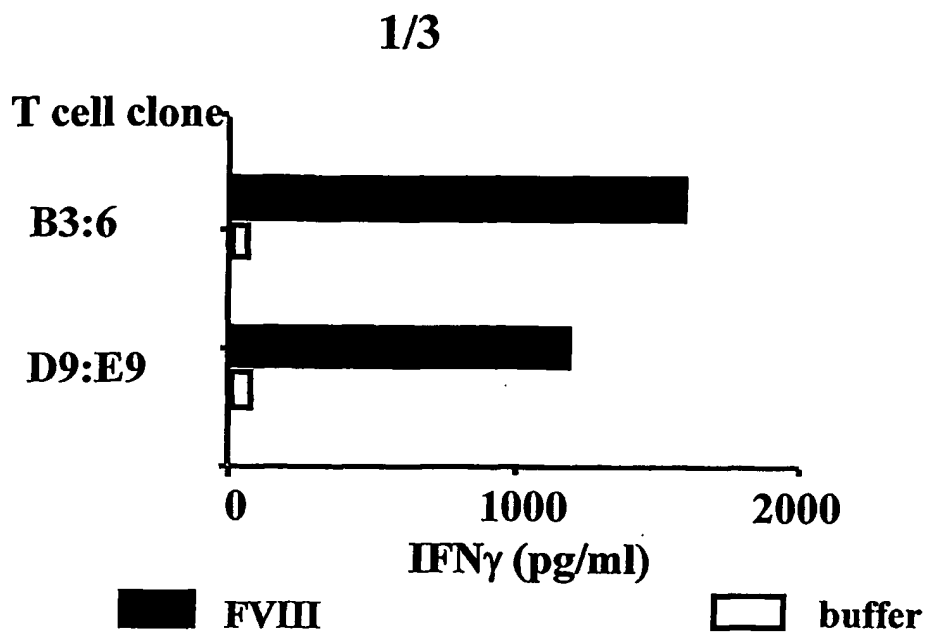
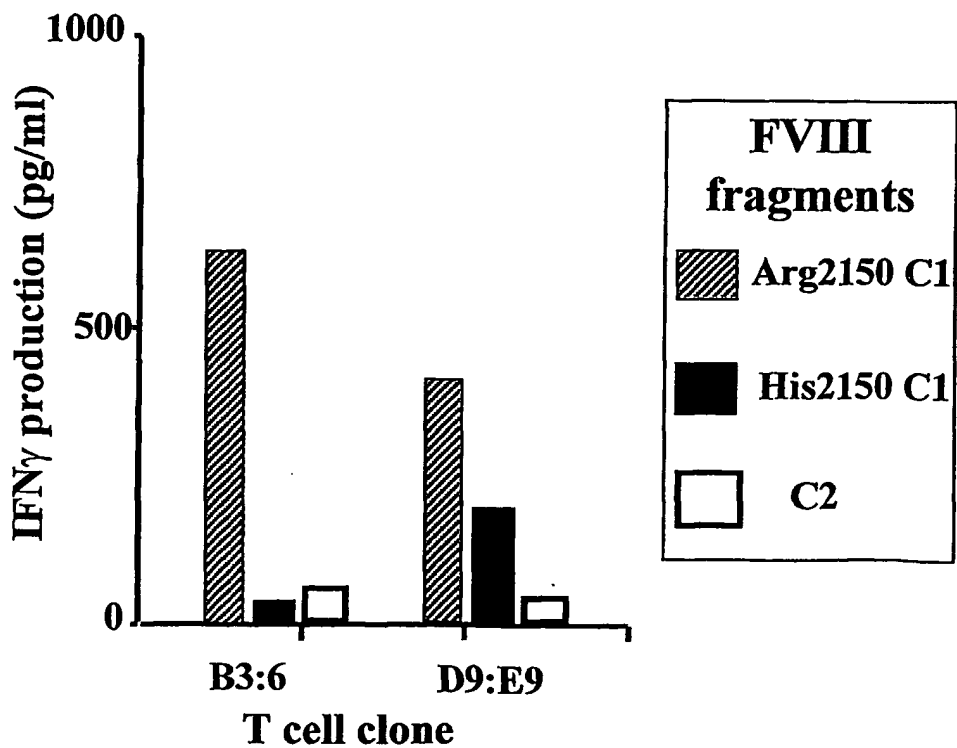
105. A method according to claim 100, wherein the said disease is haemophilia A.

106. Use of a recombinant point-mutated factor VIII light chain molecule
5 according to claim 89 or 90 as a template to determine another modification of
the factor VIII molecule is able to reduce the clearance, e.g. to increase the
half-life time, of factor VIII in plasma.

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20

**Figure 1****Figure 2**

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Amino acid residues T cell clone

	2140	2150	2160	B3:6	D9:E9
	:	:	:		
[SEQ ID NO:1]	IFNPPIIARYIRLHPT			-	-
[SEQ ID NO:2]	IIARYIRLHPTHYSIRST			++	++
[SEQ ID NO:3]	ARYIRLHPTHYSIRST			+	+
[SEQ ID NO:4]	YIRLHPTHYSIRST			+	-
[SEQ ID NO:5]	RLHPTHYSIRST			-	-
[SEQ ID NO:6]	IIARYIRLHPTHYSI			+	+
[SEQ ID NO:7]	IIARYIRLHPTHY			-	-

Figure 3

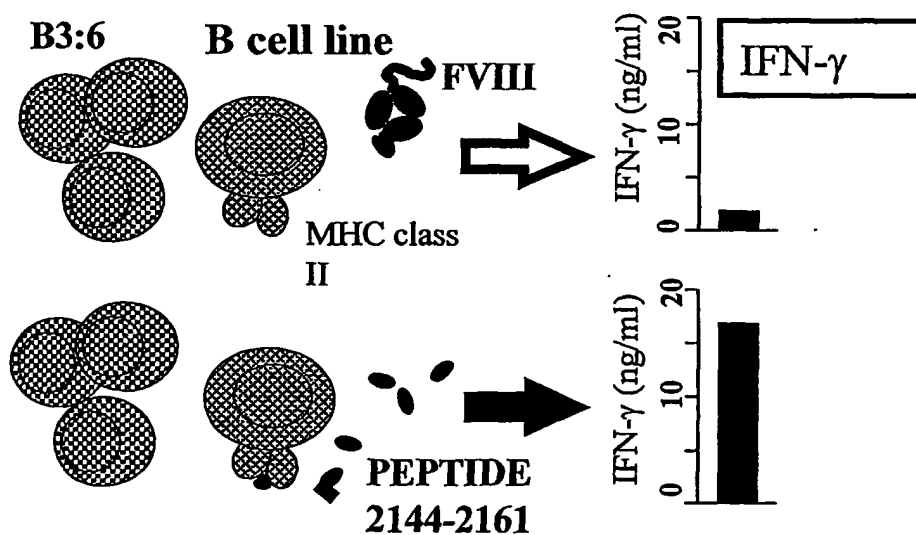


Figure 4

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Amino acid residues		cell clone		Mutation
2145	2160	B3:6	D9:E9	
:	:			
[SEQ ID NO: 8]	IIAQYIRLHP <u>THYS</u> IRST	++	++	Arg2147Gln
[SEQ ID NO: 9]	IIARYI <u>HL</u> HP <u>THYS</u> IRST	-	+	Arg2150His
[SEQ ID NO:10]	IIARYIRLH <u>Q</u> <u>THYS</u> IRST	-	-	Pro2153Gln
[SEQ ID NO:11]	IIARYIRLHP <u>I</u> <u>THYS</u> IRST	+	-	Thr2154Ile
[SEQ ID NO:12]	IIARYIRLHP <u>THYS</u> IL <u>ST</u>	++	++	Arg2159Leu

Figure 5

SEQUENCE LISTING

<110> D. Collen Research Foundation VZW
 Saint-Remy, Jean-Marie R.
 Jacquemin, Marc

<120> Recombinant molecules with reduced immunogenicity, methods and
 intermediates for obtaining them and their use in pharmaceutical
 compositions and diagnostic tools

<130> C-2059 PCT

<150> PCT/EP 01/06297

<151> 2001-05-31

<150> UK 0127321.8

<151> 2001-11-14

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Thr Leu Phe Val Glu Phe Thr Val His Leu Phe Asn Ile Ala Lys Pro
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Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
 65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
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Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
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Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
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Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
 130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
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His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
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Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
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His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
 195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
 210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg

Page 6

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
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His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
 500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
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Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
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Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
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Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
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Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
 580 585 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
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Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
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Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
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Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
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Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
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Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
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 690 695 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
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Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
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Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Thr Arg
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Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
 755 760 765

Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
 770 775 780

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
 785 790 795 800

His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
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Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
 820 825 830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
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Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
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Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
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Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
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Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
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Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
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Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
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Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
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Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu
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Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp
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Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly
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His Thr Ala His Phe Ser Lys	Lys Gly Glu Glu Glu	Asn Leu Glu
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Gly Leu Gly Asn Gln Thr Lys	Gln Ile Val Glu Lys	Tyr Ala Cys
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Thr Thr Arg Ile Ser Pro Asn	Thr Ser Gln Gln Asn	Phe Val Thr
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Gln Arg Ser Lys Arg Ala Leu	Lys Gln Phe Arg Leu	Pro Leu Glu
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Glu Thr Glu Leu Glu Lys Arg	Ile Ile Val Asp Asp	Thr Ser Thr
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Pro Leu Ser Asp Cys Leu Thr	Arg Ser His Ser Ile	Pro Gln Ala
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Asn Arg Ser Pro Leu Pro Ile	Ala Lys Val Ser Ser	Phe Pro Ser
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Ile Arg Pro Ile Tyr Leu Thr	Arg Val Leu Phe Gln	Asp Asn Ser
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Phe	Leu	Val	Tyr	Ser	Asn	Lys	Cys	Gln	Thr	Pro	Leu	Gly	Met	Ala
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Gly Ile Lys His Asn Ile Phe	Asn Pro Pro Ile Ile	Ala Arg Tyr
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Ile Arg Leu His Gln Thr His	Tyr Ser Ile Arg Ser	Thr Leu Arg
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Met Glu Leu Met Gly Cys Asp	Leu Asn Ser Cys Ser	Met Pro Leu
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Ser Tyr Phe Thr Asn Met Phe	Ala Thr Trp Ser Pro	Ser Lys Ala
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Gln Gly Asn Gln Asp Ser Phe	Thr Pro Val Val Asn	Ser Leu Asp
2285	2290	2295
Pro Pro Leu Leu Thr Arg Tyr	Leu Arg Ile His Pro	Gln Ser Trp
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 Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu
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 gaa cat ttg gga ctc ctg ggg cca tat ata aga gca gaa gtt gaa gat 240
 Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp
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 Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu
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 Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp
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 180 185 190

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caa tat gga cag tgg gcc cca aag ctg gcc aga ott cat tat tcc gga Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly 355 360 365	1104
tca atc aat gcc tgg agc acc aag gag ccc ttt tct tgg atc aag gtg Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val 370 375 380	1152
gat ctg ttg gca cca atg att att cac ggc atc aag acc cag ggt gcc Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala 385 390 395 400	1200
cgt cag aag ttc tcc agc ctc tac atc tct cag ttt atc atc atg tat Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr 405 410 415	1248
agt ott gat ggg aag aag tgg cag act tat cga gga aat tcc act gga Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly 420 425 430	1296
acc tta atg gtc ttc ttt ggc aat gtg gat tca tct ggg ata aaa cac	1344

Thr	Leu	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser	Gly	Ile	Lys	His	
	435						440					445				
aat	att	ttt	aac	cct	cca	att	att	gct	cga	tac	atc	cgt	ttg	cac	car	1392
Asn	Ile	Phe	Asn	Pro	Pro	Ile	Ile	Ala	Arg	Tyr	Ile	Arg	Leu	His	Gln	
	450					455					460					
act	cat	tat	agc	att	cgc	agc	act	ctt	cgc	atg	gag	ttg	atg	ggc	tgt	1440
Thr	His	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg	Met	Glu	Leu	Met	Gly	Cys	
465				470					475					480		
gat	tta	aat	agt	tgc	agc	atg	cca	ttg	gga	atg	gag	agt	aaa	gca	ata	1488
Asp	Leu	Asn	Ser	Cys	Ser	Met	Pro	Leu	Gly	Met	Glu	Ser	Lys	Ala	Ile	
				485					490					495		
tca	gat	gca	cag	att	act	gct	tca	tcc	tac	ttt	acc	aat	atg	ttt	gcc	1536
Ser	Asp	Ala	Gln	Ile	Thr	Ala	Ser	Ser	Tyr	Phe	Thr	Asn	Met	Phe	Ala	
				500				505					510			
acc	tgg	tct	cct	tca	aaa	gct	cga	ctt	cac	ctc	caa	ggg	agg	agt	aat	1584
Thr	Trp	Ser	Pro	Ser	Lys	Ala	Arg	Leu	His	Leu	Gln	Gly	Arg	Ser	Asn	
	515					520					525					
gcc	tgg	aga	cct	cag	gtg	aat	aat	cca	aaa	gag	tgg	ctg	caa	gtg	gac	1632
Ala	Trp	Arg	Pro	Gln	Val	Asn	Asn	Pro	Lys	Glu	Trp	Leu	Gln	Val	Asp	
	530				535					540						
ttc	cag	aag	aca	atg	aaa	gtc	aca	gga	gta	act	act	cag	gga	gta	aaa	1680
Phe	Gln	Lys	Thr	Met	Lys	Val	Thr	Gly	Val	Thr	Thr	Gln	Gly	Val	Lys	
545				550				555						560		
tct	ctg	ctt	acc	agc	atg	tat	gtg	aag	gag	ttc	ctc	atc	tcc	agc	agt	1728
Ser	Leu	Leu	Thr	Ser	Met	Tyr	Val	Lys	Glu	Phe	Leu	Ile	Ser	Ser	Ser	
				565				570					575			
caa	gat	ggc	cat	cag	tgg	act	ctc	ttt	ttt	cag	aat	ggc	aaa	gta	aag	1776
Gln	Asp	Gly	His	Gln	Trp	Thr	Leu	Phe	Phe	Gln	Asn	Gly	Lys	Val	Lys	
			580				585					590				
gtt	ttt	cag	gga	aat	caa	gac	tcc	ttc	aca	cct	gtg	gtg	aac	tct	cta	1824
Val	Phe	Gln	Gly	Asn	Gln	Asp	Ser	Phe	Thr	Pro	Val	Val	Asn	Ser	Leu	
	595					600					605					
gac	cca	ccg	tta	ctg	act	cgc	tac	ctt	oga	att	cac	ccc	cag	agt	tgg	1872
Asp	Pro	Pro	Leu	Leu	Thr	Arg	Tyr	Leu	Arg	Ile	His	Pro	Gln	Ser	Trp	
	610				615					620						
gtg	cac	cag	att	gcc	ctg	agg	atg	gag	gtt	ctg	ggc	tgc	gag	gca	cag	1920
Val	His	Gln	Ile	Ala	Leu	Arg	Met	Glu	Val	Leu	Gly	Cys	Glu	Ala	Gln	
625				630				635						640		
gac	ctc	tac	tga													1932
Asp	Leu	Tyr														

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 <211> 643
 <212> PRT
 <213> Homo sapiens
 <400> 15

Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg
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Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg
 20 25 30

Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu
 35 40 45

Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn
 50 55 60

Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp
 65 70 75 80

Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe
 85 90 95

Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu
 100 105 110

Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp
 115 120 125

Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys
 130 135 140

Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser
 145 150 155 160

Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro
 165 170 175

Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr
 180 185 190

Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg
 195 200 205

Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys
 210 215 220

Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu
 225 230 235 240

Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu
 245 250 255
 Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His
 260 265 270
 Val Phe Thr Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn
 275 280 285
 Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala
 290 295 300
 Gly Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly
 305 310 315 320
 Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu
 325 330 335
 Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly
 340 345 350
 Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly
 355 360 365
 Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val
 370 375 380
 Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala
 385 390 395 400
 Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr
 405 410 415
 Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly
 420 425 430
 Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His
 435 440 445
 Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Gln
 450 455 460
 Thr His Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys
 465 470 475 480
 Asp Leu Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile

485

490

495

Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala
500 505 510

Thr Trp Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn
515 520 525

Ala Trp Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp
530 535 540

Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys
545 550 555 560

Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser
565 570 575

Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys
580 585 590

Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu
595 600 605

Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp
610 615 620

Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln
625 630 635 640

Asp Leu Tyr

<210> 16

<211> 1929

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..(1929)

<223> Human factor VIII light chain, A2201 deletion

<400> 16

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Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg
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ctc tgg gat tat ggg atg agt agc tcc cca cat gtt cta aga aac agg 96
Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg
20 25 30

gct cag agt ggc agt gtc cct cag ttc aag aaa gtt gtt ttc cag gaa Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu 35 40 45	144
ttt act gat ggc tcc ttt act cag ccc tta tao cgt gga gaa cta aat Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn 50 55 60	192
gaa cat ttg gga ctc ctg ggg oca tat ata aga gca gaa gtt gaa gat Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp 65 70 75 80	240
aat atc atg gta act ttc aga aat cag gcc tot cgt ccc tat tcc ttc Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe 85 90 95	288
tat tct agc ctt att tct tat gag gaa gat cag agg caa gga gca gaa Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu 100 105 110	336
cct aga aaa aac ttt gtc aag cct aat gaa acc aaa act tac ttt tgg Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp 115 120 125	384
aaa gtg caa cat cat atg gca ccc act aaa gat gag ttt gac tgc aaa Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys 130 135 140	432
gcc tgg gct tat ttc tct gat gtt gac ctg gaa aaa gat gtg cac tca Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Lys Asp Val His Ser 145 150 155 160	480
ggc ctg att gga ccc ctt ctg gtc tgc cac act aac aca ctg aac cct Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro 165 170 175	528
gct cat ggg aga caa gtg aca gta cag gaa ttt gct ctg ttt ttc acc Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr 180 185 190	576
atc ttt gat gag acc aaa agc tgg tac ttc act gaa aat atg gaa aga Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg 195 200 205	624
aac tgc agg gct ccc tgc aat atc cag atg gaa gat ccc act ttt aaa Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys 210 215 220	672
gag aat tat cgc ttc cat gca atc aat ggc tac ata atg gat aca cta Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu 225 230 235 240	720
cct ggc tta gta atg gct cag gat caa agg att cga tgg tat ctg ctc Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu 245 250 255	768
agc atg ggc agc aat gaa aac atc cat tct att cat ttc agt gga cat Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His 260 265 270	816

gtg ttc act gta cga aaa aaa gag gag tat aaa atg gca ctg tac aat Val Phe Thr Val Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn 275 280 285	864
ctc tat cca ggt gtt ttt gag aca gtg gaa atg tta cca tcc aaa gct Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala 290 295 300	912
gga att tgg cgg gtg gaa tgc ott att ggc gag cat cta cat gct ggg Gly Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly 305 310 315 320	960
atg agc aca ctt ttt ctg gtg tac agc aat aag tgt cag act ccc ctg Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu 325 330 335	1008
gga atg gct tct gga cac att aga gat ttt cag att aca gct tca gga Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly 340 345 350	1056
caa tat gga cag tgg gcc cca aag ctg gcc aga ctt cat tat tcc gga Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly 355 360 365	1104
tca atc aat gcc tgg agc acc aag gag ccc ttt tct tgg atc aag gtg Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val 370 375 380	1152
gat ctg ttg gca cca atg att att cac ggc atc aag acc cag ggt gcc Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala 385 390 395 400	1200
cgt cag aag ttc tcc agc ctc tac atc tct cag ttt atc atc atg tat Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr 405 410 415	1248
agt ctt gat ggg aag aag tgg cag act tat cga gga aat tcc act gga Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly 420 425 430	1296
acc tta atg gtc ttc ttt ggc aat gtg gat tca tct ggg ata aaa cac Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His 435 440 445	1344
aat att ttt aac cct cca att att gct cga tac atc cgt ttg cac cca Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro 450 455 460	1392
act cat tat agc att cgc agc act ctt cgc atg gag ttg atg ggc tgt Thr His Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys 465 470 475 480	1440
gat tta aat agt tgc agc atg cca ttg gga atg gag agt aaa gca ata Asp Leu Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile 485 490 495	1488
tca gat gca cag att act gct tca tcc tac ttt acc aat atg ttt acc Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Thr 500 505 510	1536
tgg tct cct tca aaa gct cga ctt cac ctc caa ggg agg agt aat gcc	1584

Trp Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala
 515 520 525
 tgg aga cct cag gtg aat aat cca aaa gag tgg ctg caa gtg gac ttc 1632
 Trp Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe
 530 535 540
 cag aag aca atg aaa gtc aca gga gta act act cag gga gta aaa tct 1680
 Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
 545 550 555 560
 ctg ctt acc agc atg tat gtg aag gag ttc ctc atc tcc agc agt caa 1728
 Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln
 565 570 575
 gat ggc cat cag tgg act ctc ttt ttt cag aat ggc aaa gta aag gtt 1776
 Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val
 580 585 590
 ttt cag gga aat caa gac tcc ttc aca cct gtg gtg aac tct cta gac 1824
 Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp
 595 600 605
 cca ccg tta ctg act cgc tac ctt cga att cac ccc cag agt tgg gtg 1872
 Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val
 610 615 620
 cac cag att gcc ctg agg atg gag gtt ctg ggc tgc gag gca cag gac 1920
 His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp
 625 630 635 640
 ctc tac tga 1929
 Leu Tyr

<210> 17
 <211> 642
 <212> PRT
 <213> Homo sapiens
 <400> 17

Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg
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 20 25 30
 Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu
 35 40 45
 Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn
 50 55 60
 Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp
 65 70 75 80

Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe
 85 90 95

Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu
 100 105 110

Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp
 115 120 125

Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys
 130 135 140

Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser
 145 150 155 160

Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro
 165 170 175

Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr
 180 185 190

Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg
 195 200 205

Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys
 210 215 220

Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu
 225 230 235 240

Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu
 245 250 255

Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His
 260 265 270

Val Phe Thr Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn
 275 280 285

Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala
 290 295 300

Gly Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly
 305 310 315 320

Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu
 325 330 335

Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly
 340 345 350

Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly
 355 360 365

Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val
 370 375 380

Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala
 385 390 395 400

Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr
 405 410 415

Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly
 420 425 430

Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His
 435 440 445

Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro
 450 455 460

Thr His Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys
 465 470 475 480

Asp Leu Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile
 485 490 495

Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Thr
 500 505 510

Trp Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala
 515 520 525

Trp Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe
 530 535 540

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
 545 550 555 560

Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln

565

570

575

Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val
580 585 590

Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp
595 600 605

Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val
610 615 620

His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp
625 630 635 640

Leu Tyr

<210> 18
<211> 6999
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> (1)..(6999)
<223> Human Factor VIII P2153Q

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aacacctcag tcgtgtacaa aaagactctg tttgtagaat tcaoggttca ccttttcaac 180
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tatgatacag tgggtcattac acttaagaac atggcctccc atcctgtcag tcttcatgct 300
gttgggtgat cctactggaa agcttctgag ggagctgaat atgatgatca gaccagtcaa 360
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gaagggagtc tggccaagga aaagacacag accttgcaaa aatttatact actttttgct 600
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tctctgccag gtctgattgg atgccacagg aaatcagttc attggcatgt gattggaatg 780
ggcaccactc ctgaagtgca ctcaatattc ctogaaggtc acacatttct tgtgaggaac 840

catcgccagg	ogtccttgga	aatctcgcca	ataactttcc	ttactgctca	aacactcttg	900
atggaccttg	gacagttttot	actgttttgt	catatctctt	cccaccaaca	tgatggcatg	960
gaagcttatg	tcaaagtaga	cagctgtcca	gaggaacccc	aactacgaat	gaaaaataat	1020
gaagaagcgg	aagactatga	tgatgatctt	actgattotg	aaatggatgt	ggtcagggtt	1080
gatgatgaca	actctccttc	ctttatccaa	attcgctcag	ttgccaagaa	gcacccataa	1140
acttggttac	attacattgc	tgctgaagag	gaggactggg	actatgctcc	cttagtcctc	1200
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agcattggag	cacagactga	cttcctttct	gtcttctctt	ctggatatac	cttcaaacac	1980
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tcgatggaaa	accaggtctt	atggattctg	gggtgccaca	actcagactt	tcggaacaga	2100
ggcatgaccg	ccttactgaa	ggtttctagt	tgtgacaaga	acactgggtga	ttattacgag	2160
gacagttatg	aagatatttc	agcatacttg	ctgagtataa	acaatgccat	tgaaccaaga	2220
agcttctccc	agaattcaag	acaccctagc	actaggcaaa	agcaatttaa	tgccaccaca	2280
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